Mephedrone

a single-dose administration study to determine human pharmacokinetics after nasal insufflation and to detect mephedrone and its metabolites in novel biological matrices

Czerwinska, Joanna

Awarding institution:
King's College London

Mephedrone: a single-dose administration study to determine human pharmacokinetics after nasal insufflation and to detect mephedrone and its metabolites in novel biological matrices

Joanna Czerwinska

In partial fulfilment of the requirements for the award of Doctor of Philosophy in Analytical Toxicology

2019

Supervisors:

Dr Vincenzo Abbate

Prof Paul Dargan

Department of Analytical, Environmental & Forensic Sciences

King's College London,

London, UK

Abstract

Mephedrone is a synthetic cathinone known for its psychostimulant properties. Despite its ban in the United Kingdom in 2010, mephedrone use in London remains popular and there are reports describing an increasing problem of the drug being injected. Nevertheless, there is little known about the distribution of mephedrone and its metabolites in humans as only two controlled mephedrone administration studies and one dose-finding pilot study have been previously reported. In recent years, there has been a growing interest in the use of alternative biological matrices for determining drug abuse. The collection of these samples is usually non-invasive, fast and cost effective which allows for drug testing in the workplace, by the roadside and in addiction treatment centres.

A single dose administration study of 100 mg mephedrone hydrochloride via nasal insufflation to six healthy male volunteers was performed to determine the distribution and pharmacokinetics of mephedrone and its metabolites in conventional (whole blood, plasma, urine) and alternative (oral fluid, fingerprint sweat, dried blood spots, head hair) biological matrices. Samples were collected at different timepoints after mephedrone administration and were analysed for the presence of mephedrone, dihydro-mephedrone (DHM), nor-mephedrone (NOR), hydroxytolyl-mephedrone (HYDROXY), 4-carboxy-mephedrone (4-CARBOXY) and dihydro-nor-mephedrone (DHNM) by validated liquid chromatography-tandem mass spectrometry methods.

All analytes were detected in whole blood and plasma, where 4-CARBOXY reached the highest concentration. The mean T_{max} for mephedrone (55.0 ± 18.2 min in whole blood and 52.5 ± 20.7 min in plasma) correlated well between both matrices, indicating rapid absorption of the drug after nasal insufflation. Other analytes had a more delayed T_{max} but were all detected up to 6 h in both matrices, with mephedrone also being detectable on Day 2 in one participant in whole blood. Mephedrone had a mean half-life of 2.12 ±

0.33 h and 1.98 ± 0.30 h in whole blood and plasma, respectively. In addition, statistical analysis showed that median whole blood to plasma distribution ratios, reported here for the first time, were statistically different from 1 (unity) for mephedrone (median: 1.11), DHM (median: 1.30) and NOR (median: 0.765). Chiral analysis revealed that R-mephedrone reached higher concentrations than S-mephedrone in whole blood and had comparable pharmacokinetic parameters to total mephedrone. It has been shown that the two enantiomers of mephedrone exhibit different pharmacokinetic profiles in humans, but the clinical significance of this finding is not yet fully understood. In urine, 4-CARBOXY and DHNM were the only metabolites detectable on Day 3, making them promising markers of mephedrone use.

In the alternative biological matrices, mephedrone metabolites were detected for the first-time in head hair one month after mephedrone administration. Calculated NOR:mephedrone and DHNM:mephedrone ratios were 0.19 (n=1) and 0.21 (n=1), respectively. However, sample size was too small to suggest robust metabolite to mephedrone ratios that would differentiate external drug contamination from drug consumption. In fingerprint sweat, mephedrone and NOR were detected above the limit of detection in 62% and 3.8% of all post administration samples, respectively. Inter- and intra-subject variability was observed which can be attributed to the differences in pressure applied during fingerprint deposition, the angle and duration of contact with the deposition surface coupled with an inability to control the 'amount' of collected sweat. Given these limitations fingerprint sweat may not be ideal for use in quantitative analysis until practical solutions to these problems are found. In dried blood spots, mephedrone, NOR and 4-CARBOXY were the only analytes detected in the majority of samples. In oral fluid, mephedrone and NOR were detected but their concentrations peaked earlier than in whole blood and plasma which may be due to the contamination of the oral cavity with mephedrone after nasal insufflation.

It is hoped that this work will help with interpreting results and reporting findings from the analysis of conventional and alternative biological matrices following mephedrone

use in forensic (drug-related deaths and crime) and clinical (acute drug toxicity and drug dependence) toxicology as well as in the workplace and roadside drug testing.

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Acknowledgments

Firstly, I would to thank Dr Vincenzo Abbate and Professor Paul Dargan for being wonderful supervisors, always offering guidance and support. I would also like to thank Dr Mark Parkin for all his help and patience in the first two years of my PhD. Also, many thanks to Dr Andrew Kicman for always offering support and feedback.

Many thanks to the Clinical Toxicology unit at St Thomas's Hospital for the help with the mephedrone administration study. Special thanks go to Elizabeth Biswell who was irreplaceable and helped me with training, logistics and late afternoon sample collections.

Many thanks to Professor David Cowan and the staff at the Drug Control Centre for allowing me to carry out my research in the past four years. Also, many thanks to the staff at Abbott, especially Nicole Fernandes, for the help with the mephedrone study and the LC-MS/MS analysis of oral fluid samples.

To all my fellow PhD friends for making my time in the lab and in the PhD office so enjoyable. Very special thanks to my gym buddies (Ana, Caroline, Federica, Helena and Rachel) for always keeping me company at the gym. Lastly, to Teo for donating countless samples to the project.

To my mum and dad: bez Waszego zaangażowania i wsparcia nie byłabym w stanie osiągnąć tego co osiągnęłam. Dziękuję! Lastly, to Mal for always putting a smile on my face and teasing me about how he is a doctor and I am not. Well, this has changed now!

Abbreviations

Abbreviation	Meaning
4-CARBOXY	4-carboxy-mephedrone
α-PPP	α-pyrrolidinopropiophenone
%CV	Coefficient of variation
ACN	Acetonitrile
AEBA	4-(2-aminoethyl) benzoic acid hydrochloride
AGC	Automatic gain control
APCI	Atmospheric pressure chemical ionisation
AUC	Area under the curve
A&E	Accident and Emergency Department
BBB	Blood-brain barrier
BMI	Body mass index
BZE	Benzoylecgonine
CBD	Cannabidiol
CBN	Cannabinol
CD	Circular dichroism
CE	Collision energy
CL	Clearance
CL_r	Renal clearance
CNS	Central nervous system
CO_2	Carbon dioxide
CRF	Clinical Research Facility
CYP2D6	Cytochrome P450 2D6
DA	Dopamine
DAD	Diode array detection
DART-MS	Direct analysis in real time-mass spectrometry
DAT	Dopamine transporter

DBP	Diastolic blood pressure
DBS	Dried blood spots
DCC	Drug Control Centre
DCM	Dichloromethane
DEA	Diethylamine
DESI-MS	Desorption electrospray ionisation-mass spectrometry
DFSA	Drug facilitated sexual assault
DHM	Dihydro-mephedrone
DHM-d_3	Dihydro-mephedrone-d_3
DHNM	Dihydro-nor-mephedrone
DMPK	Drug metabolism and pharmacokinetics
DNPV	N-(2,4-dinitro-5-fluorophenyl) L-valinamide
DUID	Driving under the influence of drugs
EBC	Exhaled breath condensate
EC_{50}	Half maximal effective concentration
ECG	Electrocardiogram
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EDTA	Ethylenediaminetetraacetic acid
EF	Enantiomeric fraction
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EME	Ecgonine methyl ester
ESI	Electrospray ionisation
EtOH	Ethanol
EU	European Union
FA	Formic acid
FDA	Food and Drug Administration
F/T	Freeze-thaw
GC-MS	Gas chromatography-mass spectrometry
H^1 NMR	Proton nuclear magnetic resonance
HCl	Hydrochloric acid

Hct	Haematocrit
HESI	Heated electrospray ionisation
HIV	Human immunodeficiency virus
HR	Heart rate
HRMS	High resolution mass spectrometry
HS-SPME	Headspace-solid phase microextraction
HTA	Human Tissue Act
HYDROXY	Hydroxytolyl-mephedrone
ICSS	Intracranial self-stimulation
IPA	Propanol
IS	Internal standard
k_{el}	Elimination rate constant
L-TPC	(S)-(−)-N-(trifluoroacetyl) pyrrolidine-2-carbonyl chloride
LC-HRMS	Liquid chromatography-high accuracy mass spectrometry
LC-MS	Liquid chromatography–mass spectrometry
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LLE	Liquid-liquid extraction
LLOQ	Lowest level of quantification
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Lysergic acid diethylamide
MALDI-MS	Matrix-assisted laser desorption/ionization-mass spectrometry
MAP	Mean arterial blood pressure
MDEA	3,4-Methylenedioxy-N-ethylamphetamine
MDPV	Methylenedioxypyrovalerone
MeOH	Methanol
MEPH	Mephedrone
MEPH-d_3	Mephedrone-d_3
MS	Mass spectrometry
MSM	Men having sex with men

MTPA	(R)-(−)-α-Methoxy-α-(trifluoromethyl) phenylacetyl chloride
NA	Noradrenaline
NaF/citrate buffer	Sodium fluoride/citrate buffer
NaF/KOx	Sodium fluoride/potassium oxalate
NaOH	Sodium hydroxide
NOR	Nor-mephedrone
NPS	New psychoactive substances
NRES	National Research Ethics Service
PD	Pharmacodynamics
PFPP	Pentafluorophenylpropyl
PK	Pharmacokinetics
PPT	Protein precipitation
QC	Quality control
QC High	Quality control high
QC Low	Quality control low
QC Med	Quality control medium
RBC	Red blood cells
R-MEPH	R-mephedrone
RPLC	Reverse phase liquid chromatography
RT	Room temperature
SALDI-MS	Surface-assisted laser desorption/ionization-mass spectrometry
SBP	Systolic blood pressure
SD	Standard deviation
SER	Serotonin
SERT	Serotonin transporter
SFC	Supercritical fluid chromatography
SLE	Supported liquid extraction
S-MEPH	S-mephedrone
S/N	Signal-to-noise ratio

SoHT	Society of Hair Testing
SPE	Solid phase extraction
SRM	Selected reaction monitoring
STD	Sexually transmitted diseases
$t_{1/2}$	Half-life
TFA	Trifluoroacetic acid
THC	Tetrahydrocannabinol
TMMPI	1,2,3,5-tetramethyl-4-(4-methylphenyl)-1H-imidazol-3-iumsalt
ToF	Time-of-flight
ToF-SIMS	Time-of-flight secondary ion mass spectrometry
UK	United Kingdom
ULOQ	Upper level of quantification
UPLC-TOF-MS	Ultra-high performance liquid chromatography-time-of-flight mass spectrometry
USA	United States of America
VAMS	Volumetric absorptive microsampling
VAS	Visual analogue scale

CHAPTER 1

INTRODUCTION

1.1 New psychoactive substances

The last decade has seen the emergence of new psychoactive substances (NPS), defined by the United Nations as "new narcotic or psychotropic drugs, in pure form or in a preparation, that are not scheduled under the Single Convention on Narcotic Drugs of 1961 or the Convention on Psychotropic Substances of 1971" [1,2]. NPS were designed to mimic the pharmacological effects of the traditional drugs (e.g. heroin, cannabis or amphetamine) and to avoid existing drug legislation, which has earned them the name "legal highs". Although most NPS are structural analogues of the traditional drugs, synthetic cannabinoids are a notable example of NPS which are not structurally derived from tetrahydrocannabinol (psychoactive constituent of cannabis) [3].

NPS are often sold in bright and colourful packaging labelled "bath salts", "research chemical" or "plant food" alongside the disclaimer "not for human consumption" and a list of ingredients which often does not accurately indicate their content [4]. Information about dosing, adverse effects and warnings are often missing from the packaging while active ingredients vary in their amount within same brands/suppliers [5]. NPS are often adulterated with other drugs of abuse (e.g. amphetamine or cocaine), pharmaceuticals, herbal blends or contain synthetic impurities, such as unreacted starting materials or reaction by-products [5,6].

NPS represent a diverse family of compounds which sometimes overlap in terms of their pharmacological effects but is usually divided into several groups as shown in Figure 1-1. The total number of newly reported NPS peaked in 2014 when 101 new compounds, predominantly synthetic cathinones and cannabinoids, were reported to the European

Union (EU) Early Warning System run by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). The number dropped to 55 in 2018 which indicates fewer new compounds being reported, however, it is not necessarily correlated to a decrease in overall NPS availability. By the end of 2018, more than 730 NPS were monitored by EMCDDA, 55 of which were detected for the first-time in Europe in 2018 [7]. Moreover, almost 10,000 more seizures of synthetic cannabinoids were reported in 2016 than in 2015, making this group of drugs most frequently seized. Synthetic cathinones were the most seized NPS by quantity (1.9 tonnes in 2016 compared with 1.8 tonnes in 2015) and the second most frequently seized type of NPS, amounting to over 23,000 seizures [8].

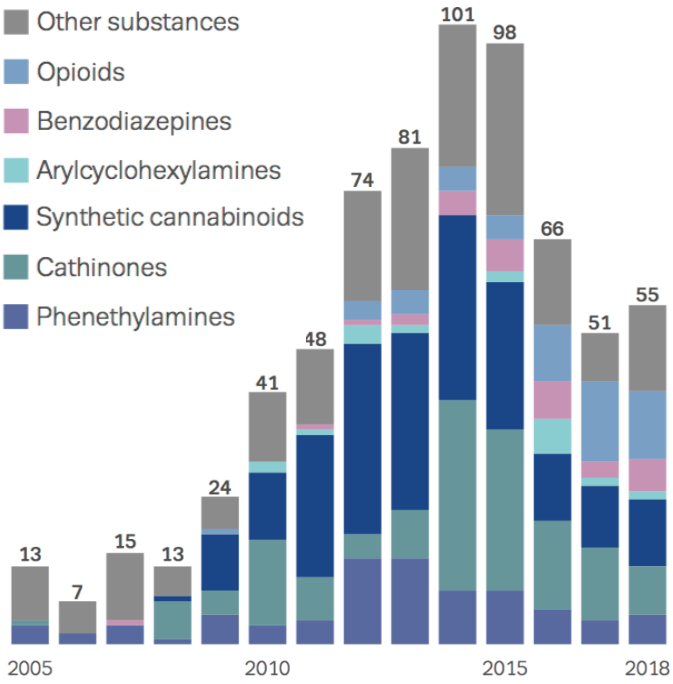


Figure 1-1. Numbers and categories of new psychoactive substances submitted to the EU Early Warning System for the first-time between 2005 and 2018; adapted from the European Drug Report 2019 [7]

A concerning trend showing the number of new synthetic opioids increasing every year can also be observed in Figure 1-1. The scale of the problem is not as serious as in the United States of America (USA), however, 11 new opioids were reported for the

first-time in Europe in 2018 compared to 9 in 2016 [9]. Seizures of opioids have also increased from 1.8 litres in 2015 to 4.6 litres in 2016.

1.1.1 Legal responses

Legal responses to the emergence of NPS differed across the globe. The UK Government introduced the Psychoactive Substances Act in 2016 which controls all substances capable of producing psychoactive effects that are not already controlled by the Misuse of Drugs Act 1971 [10]. However, it is not clear how the psychoactivity could be proved without the data from human clinical trials or controlled administration studies. Under the Psychoactive Substances Act 2016, possession of NPS for personal use is not an offence, except in the custodial institutions. Similar "blanket bans" were introduced in other European countries, such as Poland [11] and the Republic of Ireland [12]. New Zealand employed a similar model where a "blanket ban" was imposed on the psychoactive drugs which have not been shown to have a low risk of harm [13]. Countries like Brazil and Australia took a different approach and have enacted generic bans based on chemical structure rather than psychoactive effects.

1.1.2 Reasons for use

Reasons for choosing NPS instead of traditional drugs can be either internal or external. Internal reasons include curiosity, often sparked by the media reports on NPS, as well as enjoyable and desired effects. External reasons include the price, availability, ease of purchasing, legal status, perceived purity and non-detectability in routine laboratory drug tests [14,15]. The latter has especially been an attractive feature to those who have to undergo mandatory drug testing (e.g. prisoners, transport workers, military personnel) [16]. In prisons, smoking synthetic cannabinoids tend to be preferred to smoking cannabis, which is detected in routine drug screens and has an easily recognisable smell that could attract attention of probation officers or prison guards [17].

Undoubtedly, information found on the Internet about new compounds, reliable online sellers, buyers' reviews as well as the ease of purchasing have contributed to NPS popularity. Online fora became places where users described their subjective effects, drug preferences, dangerous drug interactions and routes of administration [18,19]. In addition, anonymity provided to buyers and sellers on the dark web, which cannot be accessed with standard search engines, made it an appealing drug selling platform. Research shows that in September 2013, 1,031 vendors and 10,927 individual drug listings appeared on the dark web [20]. Furthermore, popular social media sites like Facebook, YouTube and picture sharing websites have also seen drug users engaging in drug-related conversations and uploading pictures and videos of their experiences [19].

The cost of NPS has been reported to be one of the more important factors driving people to use these emerging drugs of abuse. "Value for money" reported by 59% of psychostimulant users in Australia was the main motivation for taking NPS [21]. Similar results were seen in an online survey of 619 international drug users, where 66% and 56% chose synthetic cannabinoids and synthetic opioids, respectively, due to "price, legal status, availability and non-detectability in screening tests" [22]. *Brunt et al.* have shown that the cost of NPS per gram differed considerably between countries, such as Poland, Czech Republic, UK, France and the Netherlands in 2014/15 [23]. For example, synthetic cathinones were the cheapest in Poland whereas synthetic cannabinoids were least expensive in the UK.

Legal status of NPS was a more motivating factor for the new drug users than for the long-term drug users who have established contacts with drug suppliers [21]. According to a survey conducted by *Sutherland et al.*, legality and availability were the two predominant driving forces for use of synthetic cannabinoids compared to other types of NPS. However, the introduction of the Psychoactive Substances Act in 2016 in the UK imposed a "blanket ban" on "legal highs" [17,24]. As a result, "head shops" and online shops with a domain location in the UK closed down while other remained open but stopped selling NPS [10].

1.1.3 Analytical challenges

With immunoassays lacking discriminating power to accurately identify NPS due to cross-reactivity, sensitive and selective liquid chromatography-mass spectrometry (LC-MS) based on the initial intelligence from the material safety data sheets is a technique of choice for drug testing in many forensic laboratories. However, non-targeted approaches (e.g. high-resolution mass spectrometry) can also be of value in determining a potential molecular formula and/or a chemical structure. Moreover, the use of ion ratios and UV spectra may be further employed to identify isomers in biological samples.

The influx of constantly changing NPS poses a challenge to the drug testing companies which are constantly under pressure to update their assays. While many NPS may no longer be present on the market by the time methods have been re-validated, other drugs, such as mephedrone, remain popular. In addition, unavailability of certified reference standards or their prohibitive costs further delay development of analytical techniques, making it difficult to keep pace with the ever-changing nature of NPS [25]. The absence of reference standards also hinders prompt identification of new NPS which may be present in complex mixtures/matrices or may exist as positional isomers or stereoisomers.

Because animal and human administration studies require ethical approvals, information about *in vivo* metabolism of NPS and their pharmacokinetics is often unknown. As a result, informative data is usually derived from less reliable sources, such as *in vitro* metabolism studies, analysis of samples collected from hospitalised patients or post-mortem samples. However, these approaches do not provide reliable information about human drug metabolism, which is vital when metabolites have to be targeted because the parent drug is rapidly eliminated from the body [26].

1.1.4 Synthetic cathinones

Synthetic or substituted cathinones are chemical analogues of cathinone (Figure 1-2), which is naturally present in the leaves of *Catha edulis* (Khat). Synthetic cathinones are one of the largest groups of NPS, accounting for 24% of all seized NPS in 2017 [7]. They cross the blood-brain barrier (BBB) and exert stimulating and sympathomimetic effects on the central nervous system (CNS) [27]. Adverse effects include elevated blood pressure, heart arrhythmia, palpitations, dizziness, light-headedness, paranoia, tachycardia, short-term memory problems, vomiting, sweating and migraines [28,29]. Mephedrone is one of the most popular synthetic cathinones.

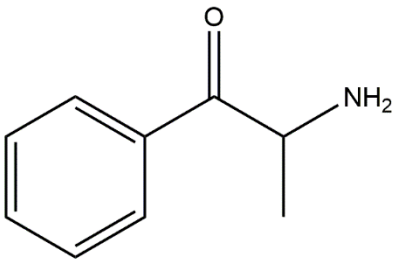


Figure 1-2. Chemical structure of cathinone

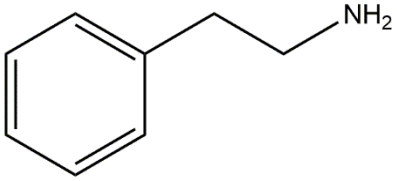


Figure 1-3. Chemical structure of phenethylamine

1.2 Mephedrone

Mephedrone (4-methyl methcathinone; 4-methylephedrone; 4-MMC; “meow meow”; “meph”; “bubble”; “M-Cat”; “drone”) is a synthetic cathinone (a β-keto amphetamine derivative) known for its psychostimulant properties [30–32]. Addition of the ketone functionality increases the overall polarity of cathinones compared to phenethylamines (Figure 1-3). Figure 1-4 shows the generic structure of a cathinone derivative and possible substitution patterns.

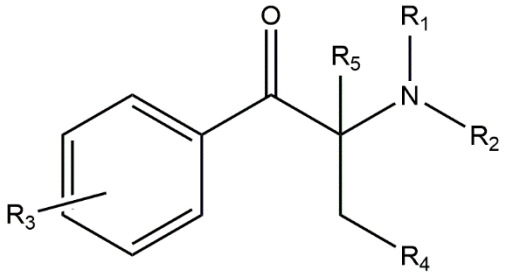


Figure 1-4. Generic structure of a cathinone derivative

The most common substitutions at the N-terminus include methyl (methcathinone) or ethyl (ethcathinone) groups or a pyrrolidine ring (α-pyrrolidinopropiophenone or α-PPP in short). In addition, substitutions on the phenyl ring lead to the formation of mephedrone (methyl group at the 4' position on the ring), 3-methylmethcathinone (methyl group at the 3' position on the ring) or methylone (dioxolane ring attached to the phenyl ring). The addition of both dioxolane (methylenedioxy) and pyrrolidine rings results in methylenedioxypyrovalerone (MDPV) and its analogues. Figure 1-5 shows chemical structures of a panel of synthetic cathinones, presenting a range of substitution patterns.

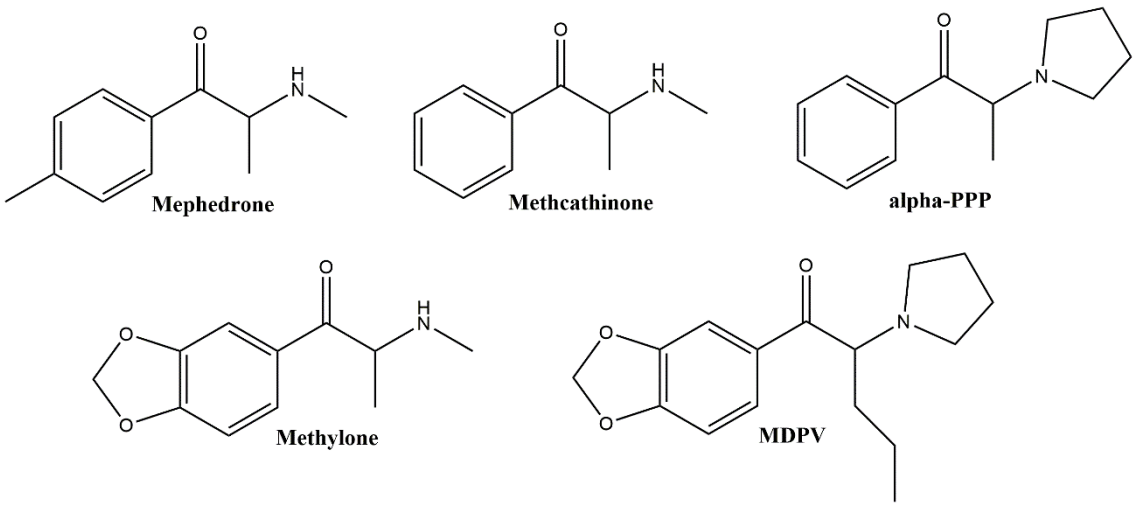


Figure 1-5. Chemical structures of selected synthetic cathinones

1.2.1 Synthesis and by-products

Even though mephedrone use in Europe began in 2009, its synthesis was first described in 1929 [33]. The main synthetic route involves α-bromination of 4-methylpropiophenone and a subsequent reaction with methylamine hydrochloride and triethylamine, the latter acting as an acid scavenger (Figure 1-6a). The resulting mixture is treated with gaseous or aqueous HCl and is recrystallised to yield mephedrone hydrochloride. Yields of 30% [34] and 51% [35] have been reported. As shown in Figure 1-6b, another potential synthetic pathway involves a one-step oxidation of 4-methylephedrine with potassium permanganate, a synthetic route also used for the preparation of methcathinone [32,33]. Inadequate purification of the product can lead to manganese poisoning which has been reported in methcathinone users [36–40]. Both methods produce racemic mixtures, but only one enantiomer of mephedrone can be produced if a single enantiomeric form of 4-methylephedrine is used [32]. As shown in Figure 1-7, a stereoselective synthesis of (S)-mephedrone via Friedel-Crafts acylation carried out with (S)-N-trifluoroacetylalanyl chloride and aluminium chloride ($AlCl_3$) can also be performed [41]. Even though stereoselective synthesis is possible, "street mephedrone" has been sold as a racemic mixture [42], which is most likely attributed to the simplicity of the synthesis and the availability of the precursors.

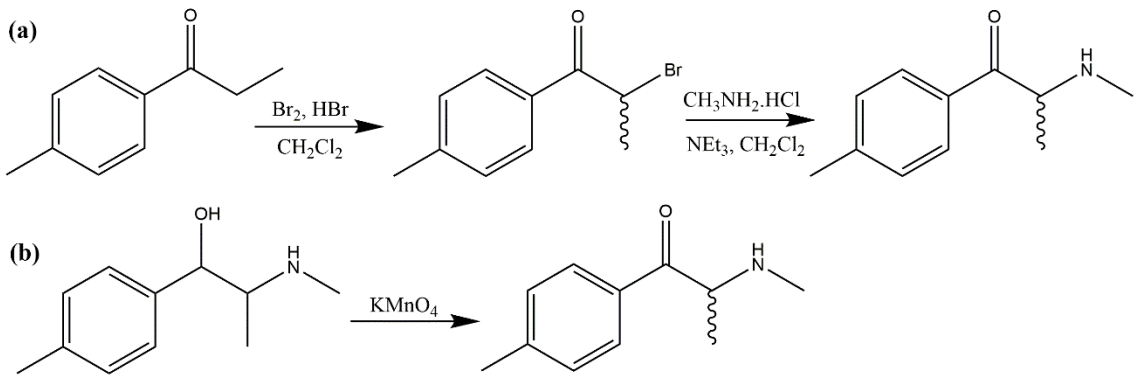


Figure 1-6. Synthesis of a racemic mephedrone via α-bromination (a) and oxidation (b)

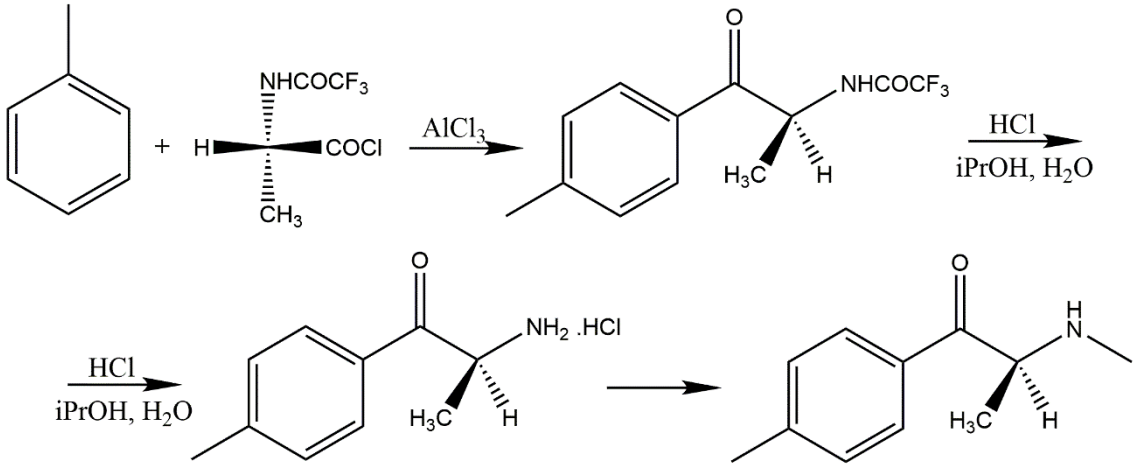


Figure 1-7. Stereoselective synthesis of (S)-mephedrone

Information about synthetic mephedrone impurities or reaction by-products, which could link a clandestine lab to the type of produced drugs, is limited. A recent publication reported the formation of precipitate following addition of diethyl ether to the crude mephedrone product. Initially, the precipitate was thought to be mephedrone itself, but it was later confirmed to also contain 1,2,3,5-tetramethyl-4-(4-methylphenyl)-1H-imidazol-3-ium salt (TMMPI) [43]. Another study identified thirteen mephedrone pyrolysis products formed during smoking. The major components were iso-mephedrone, 4-methylpropiophenone (precursor in mephedrone synthesis), 4-methylphenylacetone, two hydroxylated oxidation products, two pyrazine derivatives, N-methylated mephedrone and a diketone. Amongst minor compounds, α-chloro ketones were identified which are known respiratory irritants [44].

1.2.2 Availability and legal response

Mephedrone was first detected in the UK in 2008/9 and rapidly gained popularity. The 2013/14 Crime Survey for England and Wales reported mephedrone to be the fourth most frequently used substance amongst all recreational drugs [45]. This was reflected in the number of people seeking support for problematic and/or dependent mephedrone use, which increased from 839 in 2010/11 to 1,630 in 2012/13 [46].

Even though EMCDDA reported seizures and detection of mephedrone in 28 European and neighbouring countries, data on mephedrone prevalence and use from outside the UK is scarce [47]. *González et al.* conducted a cross-sectional survey with 230 NPS users in Spain in 2010 and 2011 [48]. The most frequently used substances were hallucinogenic phenethylamines and cathinones, including mephedrone (35.2% of surveyed NPS users). Mephedrone use was also reported on other continents. In the USA, 1.1% of clubgoers in New York City between May and October 2012 used mephedrone [49]. In Australia, one-fifth (21%) of 693 recreational users of MDMA (commonly known as "ecstasy") reported lifetime use of mephedrone and 17% took the drug in the six months preceding the interview conducted in 2010 [50].

Research shows that mephedrone's popularity was largely attributed to its price, availability and purity rather than its legal status. In semi-structured interviews carried out in 2010 none of the 23 mephedrone users pointed to its legality as a motivation for use [51]. Similar findings were reported by *Corazza et al.* who found that legal status of mephedrone did not influence drug choice of 52.9% of 446 UK students surveyed [52].

The emergence and sudden popularity of mephedrone in 2009 has also been attributed to the low purity and reduced availability of MDMA and cocaine powder [53]. Reported seizures of MDMA in west and central Europe fell from 1.5 tonnes in 2007 to 0.3 tonnes in 2008 [54]. At the same time the decline in drug purity was reported in the EU, where only 40% of "ecstasy" tablets contained MDMA-like substances in the first half of 2009 in the Netherlands [55], and the all-time low purity of seized cocaine was reported [56]. Moreover, Google searches for the word "mephedrone" peaked in the first quarter of 2009 compared to search requests for "MDMA" (Figure 1-8). A preference for "legal highs" started to emerge, with poly-drug users explaining the shift to be due to low purity of cocaine powder and "ecstasy" pills in particular [57]. However, more recent reports tend to suggest that at the time NPS, including mephedrone, did not displace or replace established drugs but rather were added to the drug repertoire [53,58].

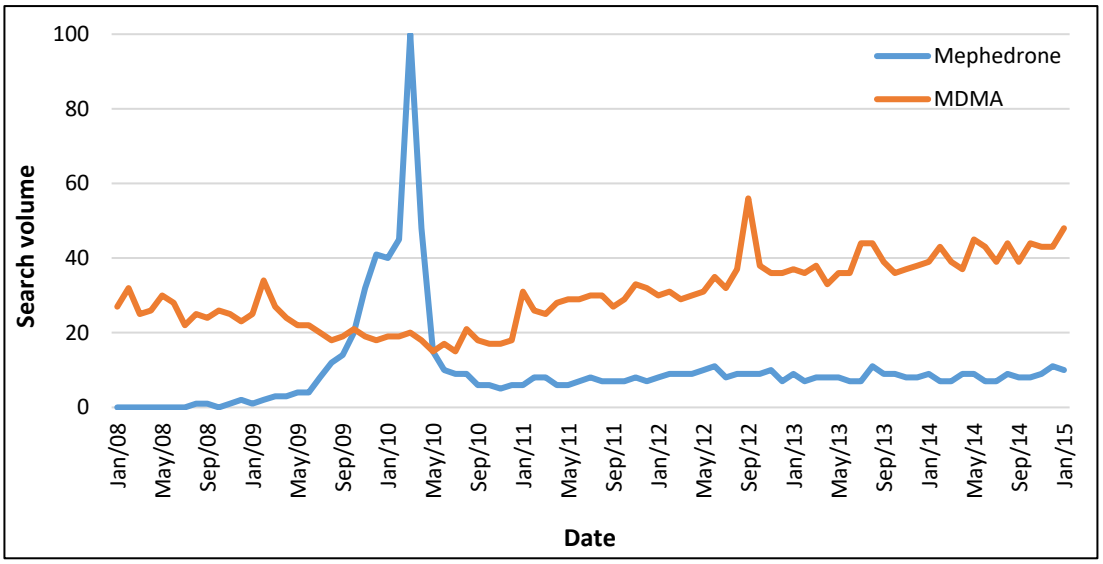


Figure 1-8. Google search queries for "mephedrone" and "MDMA" from January 2008 until January 2015

After being briefly legal in the UK, mephedrone was classified as a Class B substance and banned under the Misuse of Drugs Act, 1971 (Amendment) Order 2010 passed by the Parliament in April 2010. This was followed by an EU wide ban on mephedrone introduced by the European Council in December 2010 and a subsequent decision of the Drug Enforcement Administration to make mephedrone a controlled substance in the USA [4]. Following the 2010 ban and classification, mephedrone use declined in the UK [59]. As seen in Figure 1-9, prevalence of mephedrone use in the young generation (16-24 year olds) and in the general population (16-59 year olds) fell by 1% and 0.3%, respectively, between 2010/11 and 2011/12 [59].

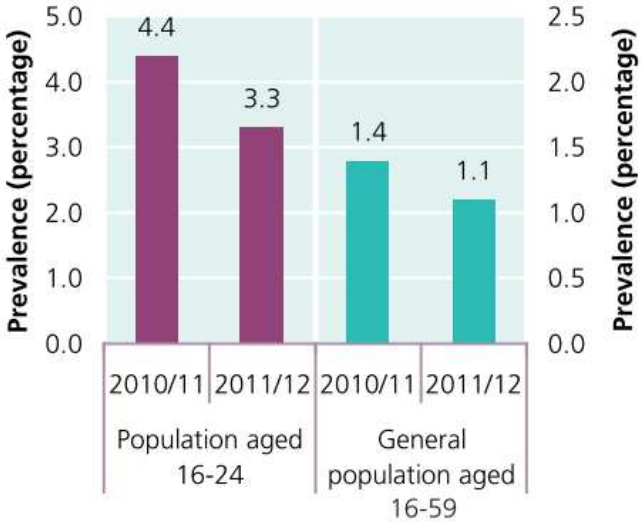


Figure 1-9. Annual prevalence of mephedrone use in England and Wales in 2010/11 and 2011/12; adapted from the United Nations World Drug Report 2013 [59]

One month after the EU ban on mephedrone in 2010, 63% of 150 surveyed mephedrone users had continued using the drug [60]. Several years after the ban, mephedrone has not completely disappeared from the market. Recent data on patients admitted to Nowowiejski Hospital in Warsaw (Poland) after a mephedrone binge started to increase steadily, reaching 100 admissions in 2017 compared to 16 in August 2010. During the mephedrone binges, which lasted on average 12.4 days, patients consumed on average 1.52 g of mephedrone a day. Almost all patients engaged in poly-drug use and took mephedrone in combination with alcohol, heroin, benzodiazepines, opioids and cannabinoids [61]. Moreover, according to the European Drug Emergencies Network published in 2015 cathinones were most frequently reported in presentations to hospitals around Europe, with mephedrone being the most common NPS [62]. In the UK, there is some evidence to suggest that mephedrone use in London remains popular and may even be increasing [63]. According to the correspondence from the Toxicology Unit at Imperial College London published in The Lancet, mephedrone was detected in 1.0% (n=34) of the Unit's death cases in 2014 and this number increased to 1.5% (n=22) in 2015. On the national scale mephedrone use among 16- to 34-year olds has decreased from 1.1% in 2014/15 to 0.3% in 2016/17 and hospitalisations due to mephedrone intoxication are also less frequent [8]. However, there is new evidence describing an

increasing problem with people injecting mephedrone which leads to higher rates of hepatitis C, human immunodeficiency virus (HIV) and lethal overdose [64]. This data indicates that mephedrone not only continues to be produced and distributed but also that the demand for the drug remains.

1.2.3 Routes of administration and dose

Mephedrone is typically sold as a white crystalline powder with a hue of yellow which is soluble in water allowing users to dissolve the powder prior to oral/rectal use or injection [33,65]. Other reports also indicate that mephedrone is supplied as tablets or in capsules filled with powder [57,66]. Most mephedrone users report "snorting" (nasal insufflation) to be the most common route of use followed by swallowing dissolved powders or powders wrapped in cigarette paper (known as "bombing") [65,67]. Even though the onset of drug action is delayed when taken orally compared to nasal insufflation, some users may want to avoid "snorting" because of reported nose bleeds and irritation associated with it [33]. Less common administration methods involve smoking as well as intravenous (see 1.2.9.3) and rectal use [65].

The initial dose of mephedrone taken by recreational mephedrone users ranges from 15 mg to more than 300 mg for oral ingestion and from 5 mg to 250 mg for nasal insufflation [66]. Because of mephedrone's short duration of action, recreational users commonly re-dose a number of times in a single session such that they use 1 g or more per session [68]. Case reports and series of acute mephedrone toxicity have generally reported use of 300-7,000 mg [33,69].

1.2.4 Human metabolism

Metabolism studies have been performed *in vitro* [70,71] and *in vivo*, both in animal (rat) [72,73] and humans [74]. As shown in Figure 1-10, the main Phase I metabolic pathways in humans include N-demethylation of the secondary amine to yield nor-mephedrone, reduction of the ketone moiety to the hydroxyl group to produce dihydro-mephedrone,

and oxidation of the tolyl moiety, leading to the formation of hydroxytolyl-mephedrone and 4-carboxy-mephedrone. A simultaneous reduction of the ketone moiety and N-demethylation of the secondary amine produces dihydro-nor-mephedrone. Phase II metabolites are produced mainly by O-glucuronidation and N-glucuronidation to form hydroxylmephedrone-3-O-glucuronide, 4-carboxymephedrone-N-glucuronide and hydroxyl-nor-mephedrone-3-O-glucuronide (see Figure 1-11) [74]. Conjugation with the succinic acid which gives rise to N-succinyl-nor-mephedrone has also been reported and its formation confirmed by the comparison with the synthesised material [74]. To the best of my knowledge, sulphur conjugation has not been reported.

Hepatic cytochrome P450 2D6 (CYP2D6) was found to be the main enzyme responsible for the metabolism of mephedrone in humans, with only a negligible contribution from other cytochrome P450 enzymes [70]. Moreover, *Olesti et al.* demonstrated that significantly altered mephedrone plasma concentrations are a result of CYP2D6 activity, with users of no or low CYP2D6 functionality being at risk of unwanted acute toxicity [75]. Cytochrome P450 enzymes are not only expressed in the liver but can also be found in the CNS which brings forward a possibility of drug metabolism in the brain and a subsequent formation of the metabolites *in situ*.

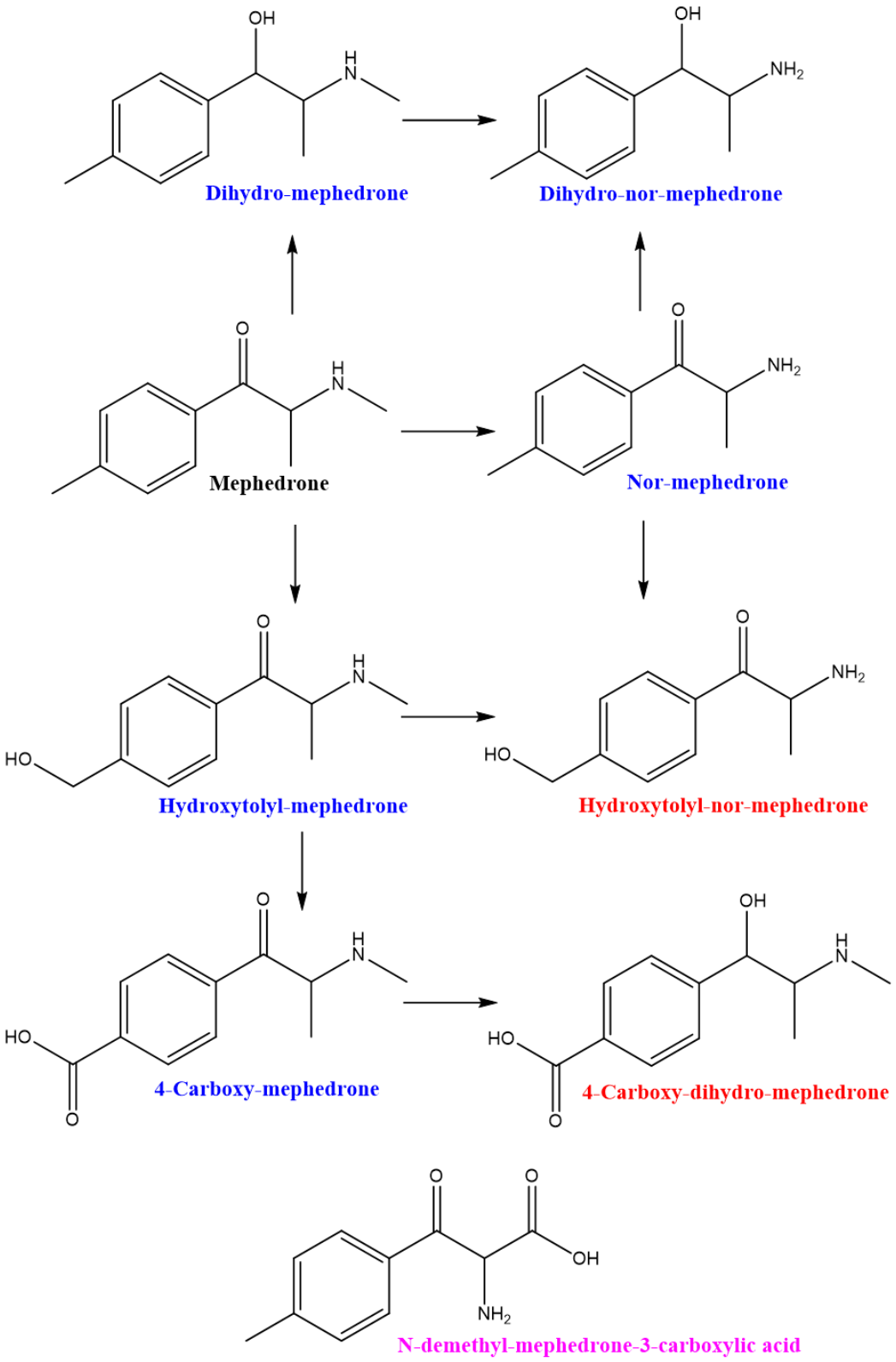


Figure 1-10. Phase I metabolites of mephedrone identified in vitro in human by Pedersen et al. [70] *and in vivo in human by Pozo et al.* [74] *Pink - metabolites identified only by Pozo et al.; Red - metabolites identified only by Pedersen et al.; Blue - metabolites identified by both*

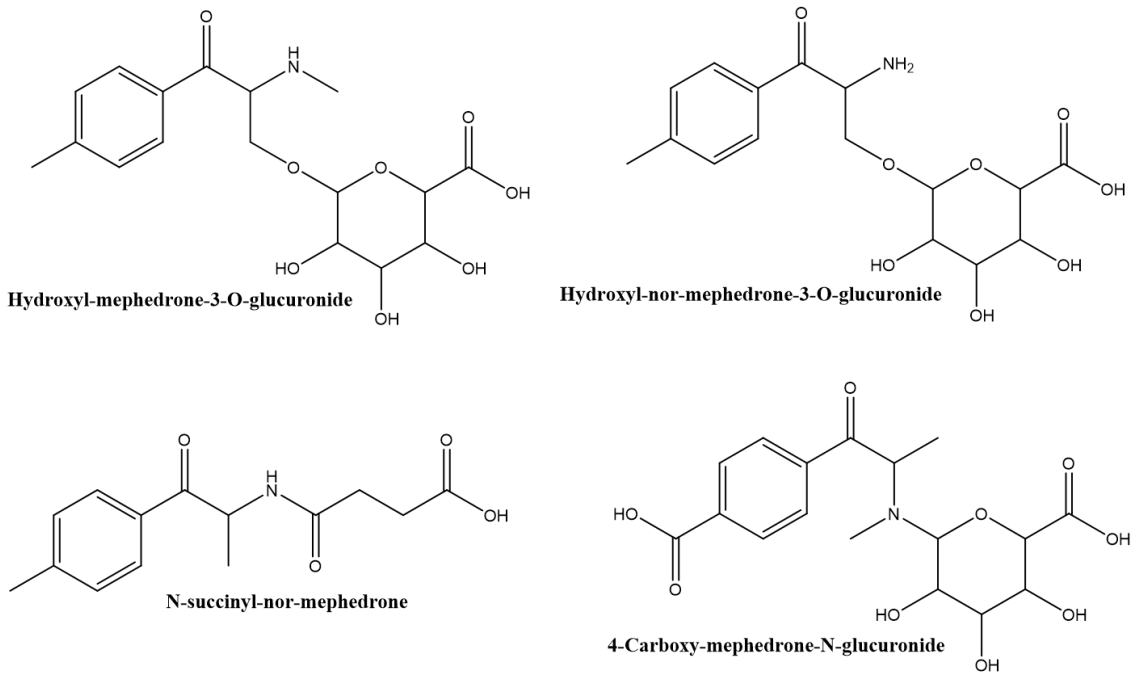


Figure 1-11. Phase II metabolites of mephedrone identified in vivo in human by Pozo et al. [74]

1.2.5 Mode of action

Studies have demonstrated that mephedrone stimulates the release of monoamines through the interaction with noradrenaline (NA), dopamine (DA) and serotonin (SER) transporters, and inhibits their reuptake [28,32,76]. A study performed on rat's tissue from the cortex and striatum showed that mephedrone had a greater affinity to DA than SER membrane transporters [77]. Researchers have also postulated that mephedrone's interaction with the SER receptor leads to entactogenic effects while the activation of DA and NA systems is responsible for the psychostimulant effects of the drug [78]. Moreover, animal studies demonstrated that mephedrone readily crosses the BBB. Following a 5 mg/kg intravenous dose, mephedrone reached the highest mean concentration (826.2 ng/mL) in a rat brain after 30 min, yielding a brain to serum ratio of 1 to 1.19 [79].

Chemical structure plays an important role in the interaction of cathinone analogues with monoamine transporters. A para-methyl group added to the cathinone structure

results in an increased potency at the serotonin transporter (SERT), decreased potency at the dopamine transporter (DAT) and an overall decrease in DAT vs SERT selectivity [80]. As seen in Table 1-1, when methcathinone was compared with mephedrone (4-methyl substituted methcathinone), methcathinone displayed a 309-fold greater selectivity at DAT in rat brain synaptosomes whereas mephedrone displayed only 2.41 DAT vs SERT selectivity [81]. Having investigated multiple substituents at the 4-position of the methcathinone scaffold, modelling studies have demonstrated that it influences drug interactions with the binding site on DAT and SERT. Large volumes of hydrophobic substituents at 4-position, like in the case of mephedrone, were shown to display greater potency at SERT [82]. Chemical modifications to the cathinone scaffold have also been shown to influence their pharmacokinetics [83]. A study done in rats investigated the effects of an α-alkyl chain length on plasma and CNS pharmacokinetics (PK). Following a 20 mg/kg subcutaneous dose of methylone ($-CH_3$), butylone ($-CH_2CH_3$) and pentylone ($-CH_2CH_2CH_3$), blood samples were collected. Significant decrease in the C_{max}, area under the curve (AUC) and half-life ($t_{1/2}$) corresponded with a decrease in the chain length. Pentylone, which is the most lipophilic of the three, reached the highest plasma concentration (C_{max} = 5,735.7 ± 799.6 µg/L), AUC ($AUC_{0-\infty}$ = 535,430 ± 41,932 µg/L x min) and displayed longest $t_{1/2}$ ($t_{1/2}$ = 253.2 ± 85.9 min) [84].

Table 1-1. In vitro release at half maximal effective concentration (EC_{50}) for methcathinone and mephedrone [81]

Drug name	DAT (nM)	SERT (nM)	DAT vs SERT selectivity
Methcathinone	12.5 ± 1.1	3860 ± 520	309
Mephedrone	49.1 ± 8.3	118 ± 26	2.41

The ability of mephedrone to cross into placenta during pregnancy has also been studied. *Strange et al.* have intraperitoneally injected mice with 5 mg/kg drug cocktail including mephedrone, methylone and MDPV. All 3 drugs were detected in the fetal brain and placenta where MDPV and mephedrone, respectively, achieved the highest

C_{max}. However, potential drug-drug interactions might have had an impact on the results [85].

Pharmacokinetic and pharmacodynamic data based on animal studies is described in 1.2.7.

1.2.5.1 Chirality of mephedrone

Due to a single chiral centre at the α-carbon, mephedrone exists as two enantiomers: (R) and (S), which are a mirror image of one another (Figure 1-12). Enantiomers usually differ in their chemical activity which may result in one enantiomer being biologically active while the other is inactive or may even produce different or adverse effects. There is some preliminary data suggesting that (R) and (S) enantiomers of mephedrone may have a different metabolic pathway and may exhibit different neurological effects. A study in rats has shown that (R)-mephedrone rather than (S)-mephedrone resulted in more stimulant-like effects due to its predominant interaction with dopaminergic receptors [86]. (R)-mephedrone displayed 50-fold higher DAT vs SERT selectivity and was responsible for increased intracranial self-stimulation (ICSS), demonstrating greater abuse-related effects. A different study looking at 56 biomarkers (including mephedrone) in wastewater reported a successful enantiomeric separation of mephedrone on a chiral column and found mephedrone in the wastewater samples to be enriched with (S)-mephedrone. This finding may suggest a possibility of an enantioselective metabolism in humans [87].

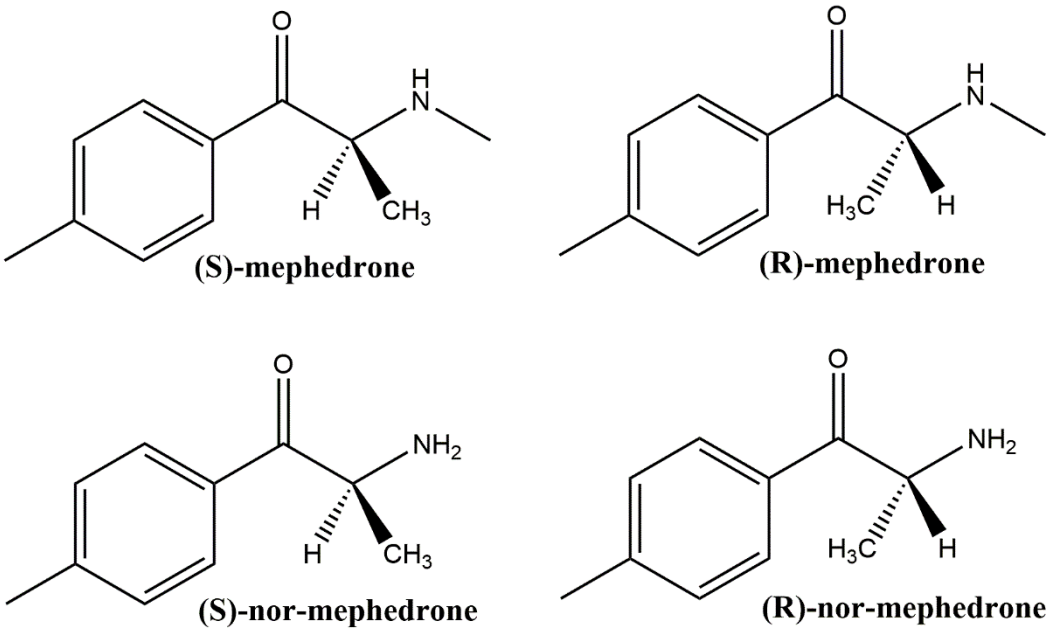


Figure 1-12. Chemical structures of mephedrone and nor-mephedrone enantiomers

A study conducted by *Castrignanò et al.* investigated metabolism of mephedrone *in vitro* (in human liver microsomes) and *in vivo* (in rat urine and human urine collected from music festivals) looking specifically at the enantiomeric ratio of (R)-mephedrone and (S)-mephedrone in the samples [88]. The researchers found human liver microsomes and human urine to be enriched with (R)-mephedrone. In rat urine, (S)-mephedrone was more predominant which might indicate differences in metabolism between humans and rats. However, there is limited information about the purity of administered mephedrone to rats as authors only state that the drug was collected from the amnesty bins at a music festival in the UK in 2014 (at the time when mephedrone was sold as a racemic mixture).

1.2.5.2 Chirality of nor-mephedrone

The stereochemistry of nor-mephedrone, which also exists as two enantiomers (see Figure 1-12), has been studied by *Hutsell et al* [81]. The authors investigated the effects of (S)-nor-mephedrone and (R)-nor-mephedrone on ICSS in rats and *in vitro* monoamine release. (S)-nor-mephedrone was more potent at DAT, NET and SERT but

(R)-nor-mephedrone displayed about 3.5 times greater DAT vs SERT selectivity. In ICSS experiments, (R)-nor-mephedrone facilitated ICSS demonstrating greater abuse-related effects whereas (S)-nor-mephedrone resulted in ICSS depression. These results follow the ICSS pattern reported for mephedrone enantiomers [86].

Mayer et al. reported that following a chiral analysis of urine samples collected from one mephedrone user, (S)-enantiomer of nor-mephedrone was approximately 2 times more abundant in urine than the (R)-enantiomer [89]. This finding is in agreement with a research published by *Castrignano et al.* who also found (S)-nor-mephedrone to predominantly exist in urine samples collected from festival urinals [88].

1.2.5.3 Mephedrone metabolites

There is little known about the effects or biological activity of mephedrone metabolites. *Mayer et al.* have investigated the effects of mephedrone, nor-mephedrone and hydroxytolyl-mephedrone on transporter-mediated uptake and release in cells and in rat brain synaptosomes [90]. All analytes were effective inhibitors of DA, SER and NA in cells expressing transporters. In synaptosomes and cells, mephedrone metabolites were shown to be substrates of monoamine transporters. In addition, *in vivo* microdialysis of rats' nucleus accumbens was performed after intravenous injections of mephedrone and its metabolite (1 mg/kg and 3 mg/kg doses). Mephedrone and nor-mephedrone elevated dialysate extracellular SER concentration by 15-fold (1 mg/kg dose) and 25-fold (3 mg/kg dose), respectively. Mephedrone also increased DA concentration after the 1 mg/kg dose whereas both mephedrone and nor-mephedrone elevated DA concentrations after the 3 mg/kg dose. Hydroxytolyl-mephedrone did not have a significant impact on SER or DA concentration at either dose. Mephedrone and nor-mephedrone were also responsible for significantly increasing locomotor activity, with mephedrone being more potent in this respect.

The permeability of the BBB for mephedrone and its metabolites has been recently studied in rats' prefrontal cortex brain area. Following a 30 mg/kg intraperitoneally

mephedrone administration, mephedrone and nor-mephedrone were the most abundant in the brain tissue 1 h post drug administration, reaching concentrations of approximately 2,400 ng/g and 5,400 ng/g, respectively [91]. These results suggest that nor-mephedrone crosses the BBB in rats and likely contributes to the psychoactive effects produced by mephedrone. Higher lipophilicity of nor-mephedrone ($logD_{7.4}$ = 1.29) and mephedrone ($logD_{7.4}$ = 1.39) compared with hydroxytolyl-mephedrone ($logD_{7.4}$ = 0.14) likely facilitates their penetration through the BBB.

1.2.6 Reasons for mephedrone use

Mephedrone users choose to take the drug because it induces a state of euphoria, increases talkativeness, enhances appreciation for music, elevates mood and reduces hostility [33,60]. In addition, mephedrone causes mild sexual stimulation and increases sex drive, which has been reported by 60% of UK clubgoers. When 947 UK clubbers were asked to compare desired effects of mephedrone with cocaine, 54.6% preferred the high associated with the use of mephedrone and 65.2% said that it was longer-lasting [65].

1.2.7 Animal data

Up to 60 mg/kg oral mephedrone has been administered to rats with no fatal toxicity reported [92]. An intravenous bolus of 10 mg/kg mephedrone resulted in the $t_{1/2}$ of 0.37 h (22.4 min) and undetectable plasma mephedrone concentrations by 4 h. After an oral dose of 30 mg/kg mephedrone, peak plasma concentration occurred within 25.8-55.8 min and mephedrone was undetectable after 6 h (Figure 1-13).

Cardiovascular effects of mephedrone have also been studied in rats and its effects were compared to the amphetamine group of stimulants. Sprague-Dawley rats given intravenous injections of mephedrone or methamphetamine showed similar increases in mean arterial blood pressure (MAP, 15-20 mmHg) and heart rate (80-90 bpm) following 1 mg/kg of methamphetamine and 3 mg/kg of mephedrone [93]. These cardiovascular effects showed a linear dose-response for MAP from 0.01 mg/kg to

9 mg/kg for mephedrone. MAP increased by approximately 10 mmHg at 0.1 mg/kg and 20 mmHg at 9 mg/kg (Figure 1-14). Heart rate increased by approximately 55 bpm at 0.1 mg/kg and 90 bpm at 9 mg/kg. Effects peaked within 2-5 min and lasted from 5 min 24 s ± 1 min 12 s (0.1 mg/kg dose, n=5) to 1 h 32 min ± 16 min (9 mg/kg dose, n=6) for MAP, and from 18 min ± 13 min 48 s (0.1 mg/kg dose) to 1 h 16 min ± 34 min (9 mg/kg dose) for the tachycardic response [93].

In animal studies comparing behavioural effects of mephedrone with MDMA, intra-peritoneal doses of 10 mg/kg of both MDMA and mephedrone produced similar behavioural effects but showed no neurotoxicity over a chronic period of administration. Mephedrone is less likely than MDMA to cross the BBB, and thus it is less likely to cause neurological/neuropsychiatric effects compared to MDMA at a given dose [93].

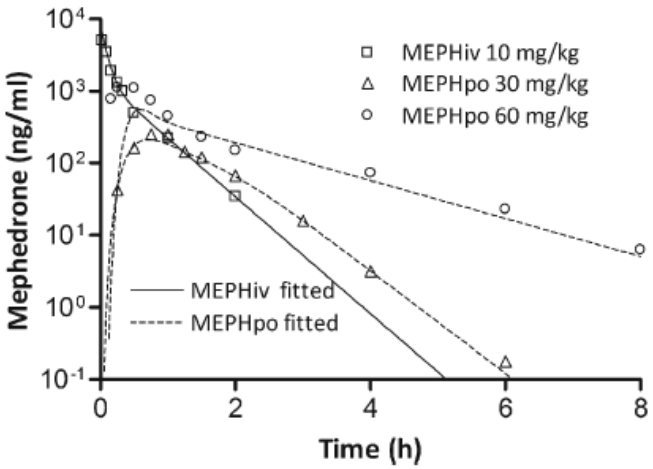


Figure 1-13. Semi-logarithmic plot of mephedrone plasma concentration after intravenous (10 mg/kg) and oral (30 mg/kg and 60 mg/kg) administrations in rats (n=4-5 rats/group); adapted from Martínez-Clemente et al. [92]

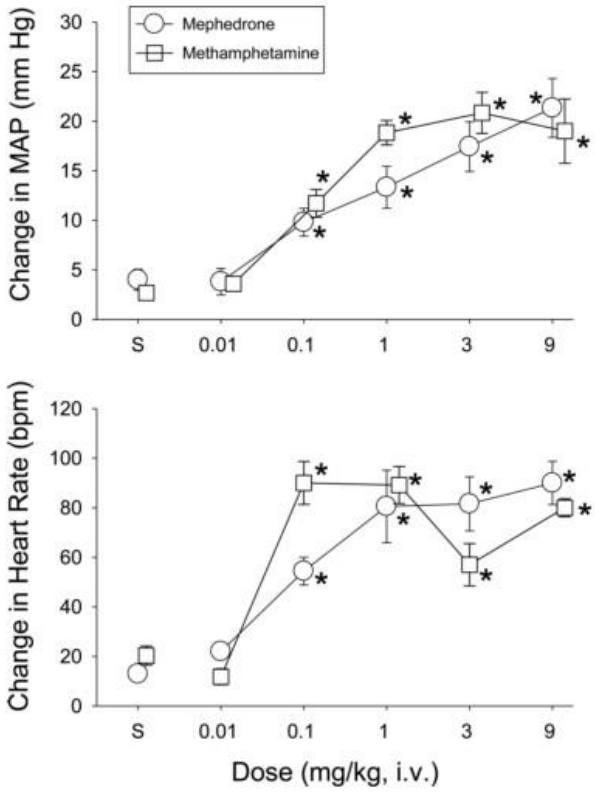


Figure 1-14. MAP and heart rate responses elicited in conscious rats by the intravenous injection of mephedrone (n=5-7) or methamphetamine (n=7) at doses of 0.01 mg/kg to 9 mg/kg. Asterisks indicate doses of mephedrone or methamphetamine that were significantly different from saline administration ($p < 0.05$); S = saline solution

Adapted from Varner et al.[93]

Bioavailability of mephedrone in humans is unknown but animal studies suggest that the absorbed fraction of mephedrone is very low. Mephedrone bioavailability was found to be approximately 10% in rats [92] and 7% in pigs [94] which was based on the investigation of mephedrone's structural isomer, 3-methyl-methcathinone.

1.2.8 Human data

At the time of this research there has only been one dose-finding pilot study (presented in a poster format) [95] and two controlled human mephedrone administration studies [96,97] published in the literature. The pilot study was a double-blind, randomised

mephedrone administration study undertaken in Spain. The study recruited 9 occasional male users of psychostimulants who were given placebo, mephedrone or MDMA on 3 different occasions, with oral doses ranging from 50 mg to 200 mg. It is important to note that both mephedrone and MDMA were obtained from police seizures and no information was provided on their purity. Therefore, it is possible that the actual doses used were lower/higher and/or that adulterants may have been present. Furthermore, there is limited data available on oral bioavailability of mephedrone and so it is difficult to determine how these oral doses compare to doses administered by the intranasal route. In the pilot study plasma and urine samples were collected and analysed for mephedrone (but not for the metabolites). Physiological effects and safety (heart rate, blood pressure, body temperature and subjective measurement of the "high" associated with drug administration) were also reported as shown in Figure 1-15. Fifty milligram and 100 mg doses of mephedrone administrated orally did not produce significant changes in heart rate (HR) and blood pressure (i.e. there was no difference with the placebo group). Mephedrone plasma concentration peaked between 1-1.5 h and the drug was undetectable after 12 h. "More pronounced" physiological effects were reported after 150 mg and 200 mg doses, however, the poster does not include data on the changes in heart rate and blood pressure for the 200 mg dose. A one hundred milligram dose resulted in increases in heart rate of 10 ± 4 bpm, systolic blood pressure (SBP) of 9.7 ± 8 mmHg and diastolic blood pressure (DBP) of 3.7 ± 5 mmHg from the baseline. As seen in Figure 1-16, PK profiles for 150 mg and 200 mg doses were similar to the ones reported for the lower doses, with peak mephedrone plasma concentration between 1-1.5 h and the drug being undetectable after 24 h.

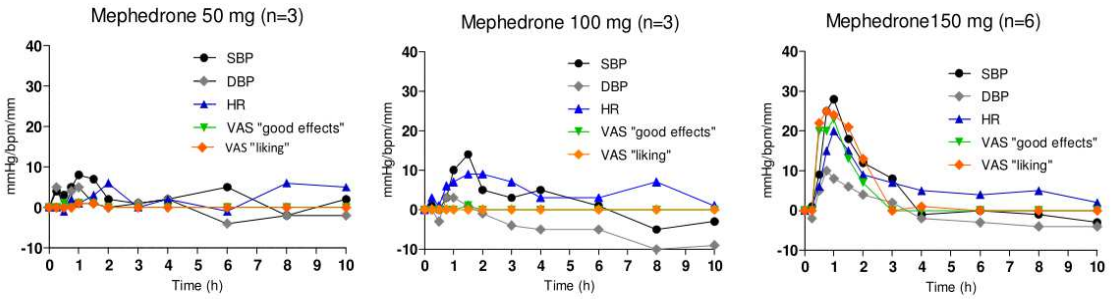


Figure 1-15. Mephedrone effects on systolic blood pressure, diastolic blood pressure and heart rate after oral administration of 50 mg, 100 mg and 150 mg; adapted from Papaseit et al. [95]

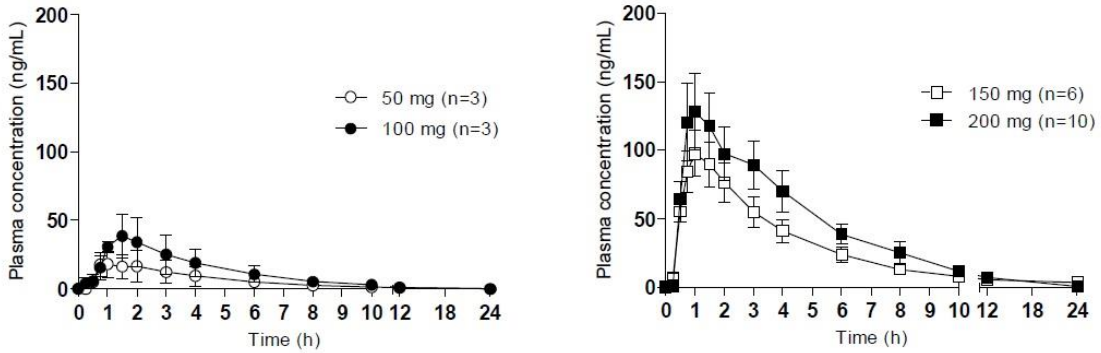


Figure 1-16. Mephedrone plasma concentrations after oral administration of 50 mg, 100 mg, 150 mg and 200 mg in human; adapted from Papaseit et al. [95]

The results from the pilot study have shown that oral doses of up to 200 mg were well tolerated. *Papaseit et al.* has subsequently conducted a double-blind, randomised, crossover, controlled administration study where 12 healthy male volunteers were orally given 200 mg of mephedrone, 100 mg of MDMA or a placebo on 3 separate occasions [96]. Mean mephedrone concentration peaked at 1.25 h in plasma (C_{max} = 134.6 ng/mL) and the drug was undetectable after 24 h. Mean $t_{1/2}$ was 2.15 h. Two hundred milligrams of mephedrone also produced increases from the baseline level in SBP (33.67 ± 10.80 mmHg), DBP (12.33 ± 9.97 mmHg) and HR (28.25 ± 17.47 bpm). Serious adverse events were not reported. Similarly to the pilot study, only plasma samples were collected and analysed for mephedrone (mephedrone metabolites were not quantified).

More recently, plasma and urine samples collected during the controlled administration study described above (*Papaseit et al.* [96]*)* have been analysed for mephedrone by GC-MS [97] and for mephedrone and four of its metabolites by LC-MS [91]. The former study demonstrated that mephedrone is rapidly absorbed (T_{max} = 1 h) and eliminated (plasma $t_{1/2}$ = 2.2 h). The latter study focused on the quantification of nor-mephedrone (NOR), 4-carboxy-mephedrone (COOH-MEPH), dihydro-mephedrone (DIHYDRO-MEPH) and N-succinyl-nor-mephedrone (SUCC-NOR-MEPH) in plasma (at 1 h, 2 h, 4 h, 6 h and 8 h) and in urine (between 0-4 h, 4-8 h, 8-12 h, 12-24 h and 24-48 h). As shown in Figure 1-17, COOH-MEPH reached the highest concentration in plasma after approximately 1.2 h followed by NOR (T_{max} = 1.5 h) and DIHYDRO-MEPH (T_{max} = 1.7 h) post drug administration. SUCC-NOR-MEPH, being a derivative of NOR, peaked much later (T_{max} = 3.7 h). COOH-MEPH and NOR were the most abundant metabolites reaching nearly the same and half of the $AUC_{0\text{-}8h}$ described for mephedrone (MEPH), respectively. Moreover, COOH-MEPH and MEPH also had similar elimination kinetics characterised by the $t_{1/2}$ of 2.2 h for MEPH and 1.9 h for COOH-MEPH. Other metabolites exhibited $t_{1/2}$ greater than 4.5 h. In urine COOH-MEPH was the most abundant analyte reaching concentrations roughly 10 times higher than those of MEPH.

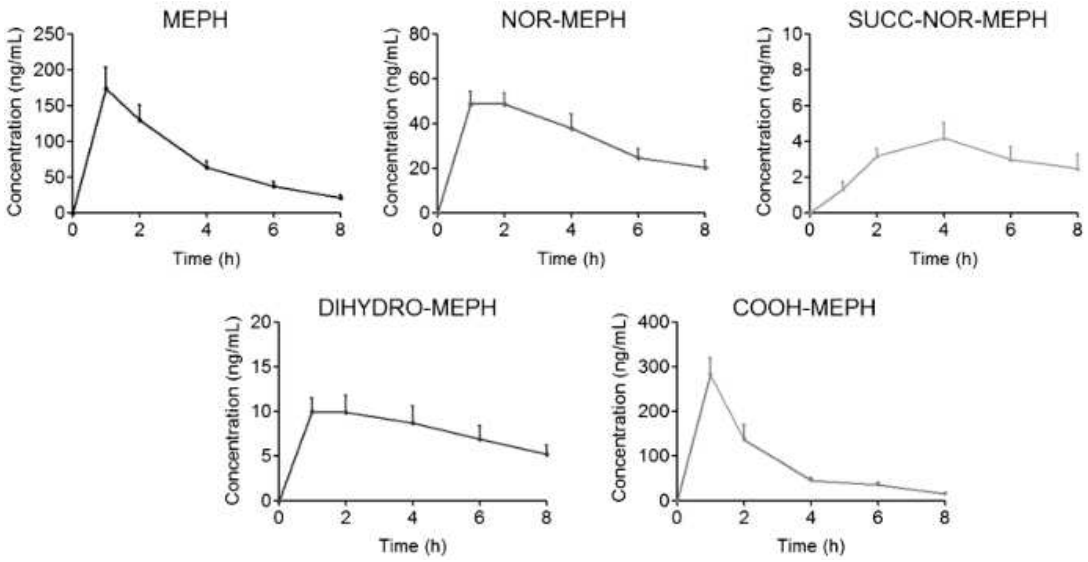


Figure 1-17. Mean concentration ± standard error of the mean for mephedrone and its metabolites in plasma collected from 6 male subjects after oral administration of 150 mg of mephedrone hydrochloride; adapted from Olesti et al. [91]

1.2.9 Harms

1.2.9.1 Adverse clinical effects

Despite its desired effects, such as increased energy, euphoria, empathy, openness and sexual arousal, 20% of mephedrone users reported significant negative effects while 28% reported a friend or an associate having an adverse reaction to the drug [98]. Unwanted effects may involve the gastrointestinal system (nausea, dry mouth, lack of appetite), cardiovascular system (vasoconstriction, elevated blood pressure, tachycardia, chest pain) or central nervous system (bruxism, dizziness, blurred vision, anxiety, agitation, seizures, hypertonia, insomnia, psychosis) [4,57,65,99].

In a total of 16 presentations to hospital with acute toxicity associated with self-reported mephedrone use, including a series of 7 with analytically confirmed mephedrone toxicity, the incidence of severe stimulant toxicity was low. Across these case reports, 13.9% had significant hypertension (> 160 mmHg systolic), 8.3% tachycardia (> 140 bpm), and no patients had hyperpyrexia (temp ≥ 39°C). In the case series of seven patients with confirmed mephedrone ingestion, mean ± standard deviation (SD) heart rate was 109.1 ± 21.8 bpm and SBP was 153.0 ± 39.6 mmHg [100]. In another published series of 57 Accident and Emergency Department (A&E) presentations with mephedrone ingestion alone or in conjunction with alcohol, 79% presented with a tachycardia > 90 bpm (mean 110.6 ± 6.7 bpm) and 74% had systolic hypertension (SBP > 130 mmHg, mean 137.6 ± 4.5 mmHg) [101].

Unusual and previously unreported conditions attributed to mephedrone have been described in the literature. A case report from the University Hospital of Gran Canaria described fingertip necrosis in a 28-year old man who was a regular recreational drug user. A few hours after an intravenous injection of an unknown dose of mephedrone, the man developed a rash, pain and edema in his thumb. Following hospitalisation and treatment, patient recovered but the mechanism of fingertip necrosis remains unknown [102]. In another report from the Colchester Hospital University NHS Foundation Trust, a

30-year old man presented to the hospital with tightness in his chest, painful neck and chest swelling following nasal insufflation of mephedrone hours earlier. The patient has been diagnosed with a spontaneous cervical emphysema attributed to the use of the drug [103]. Another case affecting the respiratory system was reported in a 17-year old man who presented to the A&E with neck pain and dysphoria. The patient had inhaled mephedrone 3 days prior to hospitalisation. X-ray and computerised tomography scans showed pneumomediastinum and subcutaneous emphysema [104].

1.2.9.2 Addiction

The dependence potential of mephedrone has not been a focus of many publications but given its pharmacology it is unlikely to cause long term physical dependency. In a web-based survey, 51% of mephedrone users found it extremely or moderately addictive and 49% experienced cravings [98]. However, 52% said that it would be easy for them to stop taking it. Moreover, a study of 1,006 individuals attending schools, universities and colleges in Scotland found only 17.5% users reporting "addiction/dependency" [105].

1.2.9.3 Chemsex

Chemsex is defined as men having sex with other men (MSM) under the influence of drugs, most commonly mephedrone, 4-hydroxybutanoic acid or crystallised methamphetamine [106,107]. In a survey of HIV-positive patients, one in ten reported engaging in the practice known colloquially as "slamming", where MSM inject drugs during sexual activities [108]. Needle sharing carries a risk of sexually transmitted diseases (STD) and viral infections, such as HIV and hepatitis C. Since 2018, a 148% increase (from 2,874 to 7,137) has been recorded in syphilis diagnoses mostly among gay and MSM community [109]. Data from the Antidote Clinic in London shows that the number of gay men injecting crystal methamphetamine or mephedrone in sexual context went up from 20% in 2011 to 80% in 2012 [110]. Seventy five percent of these users took mephedrone solely to facilitate sex though increased arousal, euphoria, confidence and disinhibition

[107,111]. "Slamming" has also been associated with psychiatric problems. In 2016 *Dolengevich-Segal et al.* reported a case of a 25-year old man presenting psychotic symptoms after "slamming" mephedrone almost every week over a 4 month period at chemsex parties [112]. The patient re-dosed 0.1-0.2 g of mephedrone diluted in physiological serum multiple times. In another study, intravenous users of mephedrone reported intense paranoia, with two of them showing violent behaviour and aggression [113].

1.2.9.4 Fatalities

Mephedrone alone or in combination with other substances was attributed to several deaths [114–117]. The first reported case was of a 18-year-old female in Sweden in 2009 who suffered cardiorespiratory arrest [118]. Post-mortem analysis of urine and blood showed presence of mephedrone only but its concentration was not reported. In 2011 in Scotland mephedrone and two metabolites (nor-mephedrone and 4-carboxy-dihydro-mephedrone) were detected in four fatal cases but due to the lack of available reference standards quantification of the metabolites was not possible. Measured mephedrone concentration in femoral blood ranged from 1.2 mg/L to 22 mg/L [116]. Fatalities attributed to a sole mephedrone use are rare but cases involving use of MDMA [119], heroin [117] or methadone [115] alongside mephedrone have also been reported.

1.2.10 Stability in biological matrices

1.2.10.1 Whole blood

The stability of mephedrone has previously been investigated in human whole blood containing different preservatives and stored under different conditions [120–123]. Mephedrone has been reported to be most stable at -20°C when preserved with acidic preservatives, such as sodium fluoride/potassium oxalate (NaF/KOx) and sodium fluoride/citrate buffer (NaF/citrate buffer). The underlying cause of its instability in

biological matrices is unknown but a previous study looking at mephedrone degradation in alkaline solution suggests the involvement of oxidants such as dissolved oxygen [124].

Studies investigating stability of mephedrone in human whole blood have been published before. *Sørensen* investigated the stability of cathinones (including mephedrone) and related ephedrines in human whole blood spiked with analytes at 100 µg/mL and preserved with NaF/KOx or NaF/citrate buffer. Samples were stored at either +4°C or +20°C for up to 5 or 6 days [121]. After 5 days of storage mephedrone was more stable at +4°C than +20°C. *Busardò et al.* reported on the stability of mephedrone in ante-mortem and post-mortem blood preserved with NaF/KOx or ethylenediaminetetraacetic acid (EDTA) [122]. Whole blood samples were spiked at 1 mg/mL and stored at -20°C, +4°C or +20°C for up to 185 days. Mephedrone was shown to be most stable in ante-mortem samples at all tested storage conditions, with -20°C being the best storage temperature. This study showed that mephedrone stability is pH dependent and acidic preservatives are better suited (6.6% vs 9.4% loss after 185 days at -20°C when preserved with NaF/KOx rather than EDTA). Johnson and Botch-Jones investigated the stability of four designer drugs (including mephedrone) stored at -20°C, +4°C or +22°C over 14 days [120]. Human whole blood (preservative not stated), plasma and urine samples were spiked at 1 µg/mL. This study showed a mean 48% reduction in mephedrone concentration in whole blood kept at +4°C for 14 days. Over the same period of time mephedrone was undetected when stored at room temperature whereas there was no measurable degradation at -20°C. The most recent study looked at the stability of mephedrone and other synthetic cathinones in bovine blood fortified with NaF/KOx at 100 ng/mL (quality control low (QC Low)) and 1,000 ng/mL (quality control high (QC High)) stored at -20°C, +4°C, +20°C and +32°C. At QC Low a complete degradation of mephedrone was observed after 11 days when stored at the elevated temperature. Degradation was much slower at +4°C and -20°C where a 20% loss was observed after 55 days and 130 days, respectively [123]. These results follow the stability pattern reported in our recent publication where mephedrone and its metabolites have been shown to be more stable at -20°C than +4°C (see Section 2.6.1.8 in Chapter 2 for more details) [125].

1.2.10.2 Urine

In urine, stability of mephedrone was investigated at pH 4 and pH 8 after sample storage at -20°C, +4°C, +20°C and +32°C over 6 months [126]. Mephedrone was more stable in acidified urine, where losses of no more than 20% were observed in frozen and refrigerated samples. Similar results have been reported by *Al-Saffar et al.* who also evaluated stability of mephedrone in urine at 1,000 ng/mL over the period of 3 months at -20°C, +6°C and +22°C [127]. Significant losses were observed at +22°C after 1 month (100% loss), 1 week (95.4% loss) and 1 day (23.2% loss). Mephedrone was stable at -20°C for up to 3 months.

1.2.10.3 Oral fluid

One stability study was carried out at 2.5 ng/mL (QC Low) and 150 ng/mL (QC High) in neat oral fluid and in buffered oral fluid collected with Quantisal® and Oral-Eze® collection devices. Samples were stored at room temperature (RT), +4°C and -20°C for up to 1 month [128]. In neat oral fluid mephedrone was most unstable at RT where it lost 91 ± 6% and 95 ± 0.3% at QC Low and QC High, respectively, compared to 61 ± 3.5% and 52 ± 2.5% losses at QC Low and QC High, respectively, at +4°C after 1 month. The use of Quantisal® and Oral-Eze® devices for oral fluid collection has greatly increased analyte stability. After 1 month at RT mephedrone lost 32 ± 11.4% at QC Low and 32.8 ± 2.4% at QC High (Quantisal® collection device), and 63 ± 11.1% at QC Low and 61 ± 0% at QC High (Oral-Eze® collection device). At -20°C mephedrone showed good stability at both concentration levels, losing no more than 5.3 ± 0.9% of its initial concertation in neat oral fluid after 1 month.

1.3 Alternative biological matrices

Urine and blood are the most commonly tested biological matrices. However, in recent years there has been an increasing interest in the use of alternative biological matrices (breath, oral fluid, dried blood spots, sweat, nails, meconium and other) for determining

and confirming drug use. The interest in the alternative biological matrices has been re-ignited in recent years thanks to the advances in hyphenated LC-MS instruments and new levels of achievable sensitivity [129].

The collection of alternative biological matrices is usually non-invasive, fast and cost effective, which allows for the workplace drug testing, assessment of driving impairment due to drug use and drug monitoring in addiction treatment centres. Alternative biological matrices are usually easier and cheaper to transport than the conventional matrices (whole blood/plasma, urine) because smaller volumes/quantities are collected and some of the matrices (head hair, dried blood spots in some cases) can be stored at ambient conditions.

1.3.1 Brief introduction to sample preparation techniques

The aim of sample preparation is to remove interferences, concentrate samples and reconstitute them in an appropriate solvent required for instrumental analysis. Even though there are many different sample preparation techniques, by far the most commonly used are protein precipitation, liquid/liquid extraction and solid phase extraction.

Protein precipitation (PPT) is a quick plasma/serum or whole blood sample clean-up technique. The most common way of precipitating proteins out of a sample is by addition of an organic solvent, usually acetonitrile, which denatures proteins resulting in their precipitation. The precipitated proteins can then be removed by either filtration or centrifugation. PPT is a fast extraction method but It does not remove other interferences, such as lipids, which may have an impact on sensitivity and contribute to matrix effects.

In liquid/liquid extraction (LLE) organic, immiscible solvent is added to the aqueous sample, creating two separate layers. Following agitation by shaking, rotating or vortex mixing, analytes of interest migrate usually from the aqueous sample matrix to the

organic layer although that is dependent on their physico-chemical properties. The organic layer, where the analytes have been extracted to, is then used for further sample manipulation. The opposite scenario where analytes remain in the original sample whilst other components of the sample are extracted to the organic layer is also possible. In this case analytes remain in the original (aqueous) sample which may not be desired if for example samples need to be quickly evaporated. Even though LLE provides some degree of sample clean-up (e.g. removal of proteins, phospholipids, salts), it may require the use of hazardous organic solvents. Moreover, emulsions may form making the layers difficult to identify, which results in incomplete analyte diffusion and difficult solvent transfers. Because LLE can be difficult to automate, a new and quick technique called supported liquid extraction (SLE) has been gaining popularity due to the 96-well plate format. Following sample loading on a SLE plate, aqueous samples soak into the highly polar support phase, concentrating non-polar analytes on its surface. The analytes are then washed off with a non-polar solvent, such as ethyl acetate or dichloromethane.

Solid phase extraction (SPE) is the most selective and powerful sample preparation technique. SPE products come in a variety of formats, such as cartridges and 96-well plates which are well suited for automation. Silica-based sorbent or polymer sorbents are widely used in SPE, which offer different polarities ranging from non-polar C18 to more polar phenyl sorbents. The sorbent is chosen in such a way to selectively target the analytes of interests and to remove matrix interferences. Before samples are loaded onto the cartridge/plate, the sorbent is conditioned first with a water-miscible organic solvent (e.g. methanol) to 'wet' the surface of the sorbent and to ensure adequate interactions. In order to maximise retention of the analytes, the sorbent is then equilibrated with a solvent similar in its composition to the samples. Samples are loaded onto the SPE media, where the target analytes are retained on the sorbent by the specifically chosen chemistry while the sample matrix flows through the sorbent to waste. SPE cartridges/plates are then washed to remove matrix interferences (salts, proteins, phospholipids and other endogenous compounds) while keeping the analytes of interest retained on the sorbent. To ensure the cleanest extract and the removal of the greatest amount of interferences, washing should be performed with the strongest

solvent which does not cause elution of the analytes. After a series of washes, analytes are eluted by disrupting interactions between the functional groups on the analytes and the sorbent. Nowadays, sorbents offering multiple retention mechanisms, referred to as “mixed mode”, are commonly used in pharmaceutical and toxicology industry for the simultaneous extraction of basic and acidic drugs. SPE offers an effective sample clean-up, can be used to extract a wide range of analytes and concentrates samples. However, SPE methods are time-consuming, more expensive than PPT or LLE and require longer method development.

1.3.2 Oral fluid

Oral fluid permits detection of a very recent drug use and does not present problems with sample adulteration which is often associated with urine collection [130,131]. Moreover, concentration of an unbound/free drug in oral fluid can often be correlated with the actual concentration of the drug in the systemic circulation, allowing impairment/intoxication to be assessed [132] (notable exceptions are drugs likely to deposit in the oral cavity, such as smoked cannabis). On the other hand, people may suffer from the dry mouth syndrome, which results in no or too little oral fluid collected for analysis and sample analysis requires sensitive instrumentation [131].

The use of oral fluid has been evaluated in the workplace drug testing [133], roadside drug testing [134–136] and drug treatment programmes [137,138]. Oral fluid has been chosen as a preferred matrix in the roadside drug testing in France [139], Belgium [140] and several states in Australia [141]. In 2014 in the UK, section 5A of the Road Traffic Act 1988 was amended to allow police officers to use approved drug testing devices (such as Securetec DrugWipe 3S®) for oral fluid collection during roadside drug testing. A positive result of the presumptive test was to be followed by a collection of a urine or blood sample at a police station [142].

1.3.2.1 Oral fluid composition

There is a difference between saliva and oral fluid. Saliva, which contains water (> 97%) and small amount of proteins, is excreted by the salivary glands whereas oral fluid is a mixture of saliva, cellular debris, food and bacteria [143]. The composition of oral fluid changes continuously throughout a day and is influenced by the circadian rhythm, disease state, emotional state and consumed food [144]. Several millilitres of saliva are usually produced every minute, but this number can be as low as zero or much higher depending on several factors. Salivation is stimulated by pain, chewing, taste or olfactory stimuli, pregnancy related hormones as well as certain drugs. On the other hand, a so called dry mouth syndrome can be caused by stress, dehydration, menopause related hormones and certain drugs [144].

Normal pH of saliva ranges from 6.7 to 7.4 but can be easily lowered by acids being released by bacteria breaking down carbohydrates originating from sweet beverages, chocolates and other products [145]. On the other hand, stimulation of the salivary glands can increase the pH to 8. Other ways of changing the pH of oral fluid include chewing a gum [146] or using collection devices which may stimulate saliva production [147]. The latter has been shown to lower concentrations of codeine by about two to six-fold [146,148] and concentrations of cocaine by about five-fold in oral fluid [149].

1.3.2.2 Drug incorporation into oral fluid

Drugs can get incorporated into oral fluid either through an active transport, passive diffusion through the phospholipid bilayer across the concentration gradient (suitable for lipid soluble and non-ionised molecules) or diffusion through pores in the membrane (molecules smaller than 300 Da) [143,144]. The ability of a drug to transition into oral fluid from blood is dependent on its pKa, lipophilicity/partition coefficient and drug-protein binding. Unlike blood/plasma, saliva does not offer much drug-protein binding capabilities and so free drugs are usually found in this matrix. Drugs of abuse usually contain a readily ionisable amine group which is characterised by a pKa value greater

than the pH of oral fluid (pH 6.7-7.4). Once a basic drug gets incorporated into oral fluid, it becomes ionised which prevents its back diffusion into the blood (pH 7.4). Basic drugs will, therefore, be present for longer and at higher concentrations in oral fluid than acidic drugs, such as cannabis (tetrahydrocannabinol and its carboxy metabolite) [137].

1.3.2.3 Correlation of oral fluid drug concentrations with whole blood and plasma

Because oral fluid is an ultrafiltrate of the interstitial fluid which contains free drugs, it should in theory represent the current concentration of the unbound drugs in the bloodstream, which could further be translated into impairment. A large body of research has been dedicated to the investigation of the correlation between oral fluid drug concentrations with whole blood and plasma [134,135,150–153]. This is most commonly done by calculating oral fluid to blood or plasma (OF/B or OF/P) drug concentration ratios from experimental results or from a theoretical model shown in Equation 1-1, where f_s (fraction of an unbound drug in saliva) is generally assumed to be negligible because of small presence of proteins in the matrix. Saliva to plasma (S/P) ratios rather than oral fluid ratios have been used in literature [146,154].

$$(1)\quad \frac{S}{P} = \frac{1 + 10^{(pH_s - pK_a)}}{1 + 10^{(pH_p - pK_a)}}\ x\ \frac{f_p}{f_s}$$

$$(2)\quad \frac{S}{P} = \frac{1 + 10^{(pK_a - pH_s)}}{1 + 10^{(pK_a - pH_p)}}\ x\ \frac{f_p}{f_s}$$

Equation 1-1. The equation showing calculations for the S/P ratio for weak acids (1) and weak bases (2), where f_p and f_s is the fraction of an unbound drug in plasma and saliva, respectively; pH_s is salivary pH; pH_p is the pH in plasma; S is the drug concentration in saliva; P is the drug concentration in plasma [155]

Neutral drugs (e.g. ethanol or antipyrine) exhibiting no protein binding will have OF/B or OF/P ratio of approximately 1. Weak acids or highly protein bound drugs, such as

caffeine or diazepam will have a ratio smaller than 1. Benzodiazepines are known to be highly protein bound in plasma [130], resulting in the concentration of these drugs in oral fluid to be low (OF/P ratio of 0.03 has been reported for diazepam [156]). The opposite trend is observed for weak bases or drugs exhibiting low protein binding, such as methadone or prilocaine. They will have the OF/P ratio greater than 1, however, falsely elevated results have been reported in cases of drugs being nasally insufflated, taken orally or smoked, resulting in a potential contamination of the oral cavity [154]. Once a drug has reached an equilibrium between oral fluid and plasma, the oral fluid to plasma drug concentration ratio will be solely dependent upon the pH of both matrices [157].

In general, drug concentration in blood tend to correlate well with oral fluid, but the strength of the correlation needs to be individually investigated for every drug. A number of controlled administration studies of cocaine [158], cannabis [150] as well as sampling of the drug users [134,153,159] have shown diverse and inconclusive results. For example, *Scheidweiler et al.* have reported that following a controlled administration, concentration of cocaine, benzoylecgonine (BZE) and ecgonine methyl ester (EME) was strongly correlated in plasma and oral fluid [158]. This was contradicted by *Langel et al.* who reported only a weak correlation for cocaine and BZE in people suspected of driving under the influence of drugs [154]. Both studies found considerable inter- and intra-individual variations and concluded that oral fluid drug concentration should not be used to infer blood concentrations of the studied drugs.

1.3.2.4 Collection devices

Decades ago, neat oral fluid was collected by spitting into a tube. The viscous fluid was, however, difficult to work with and often required centrifugation to separate food or other debris from the sample. Problems also arose with obtaining an adequate volume which would be sufficient to detect analytes of interest on the instruments used at the time [131]. Nowadays, commercial collection devices use a diluent which is mixed with oral fluid. A Certus® collection device is an example of a commercial device where oral fluid is collected onto an absorptive pad which is placed in the mouth (Figure 1-18). An

indicator changes colour to inform the user that sufficient volume has been collected. A pad is then placed in a tube containing a buffer solution, which dilutes the sample but also introduces potential matrix interferences which may impact subsequent analysis.

Figure 1-18. A Certus® device used for oral fluid collection

High inter- and intra-individual variability in reported drug concentrations in oral fluid has been observed [146,152]. Collection devices have also been shown to vary greatly in the collected volume recovered from the devices, resulting in varied recoveries of amphetamines (16-59%), opiates (33-50%), cocaine and BZE (61-97%) [146]. It is equally important to study the influence of toothpaste, mouthwash, food, beverages and commercial adulterants on drug concentrations in oral fluid. The effectiveness of a commercial device Oratect® has been extensively evaluated in respect to the above and showed that the aforementioned factors do not affect drug concentrations in oral fluid 30 min after drug use [160].

1.3.2.5 Detection of drugs in oral fluid

As drug concentrations in oral fluid are typically found in low ng/mL range, highly sensitive assays are required. Immunoassays utilising antibodies with drug class specificity are employed as a screening tool followed by a confirmatory analysis of presumptive positive samples on LC-MS or gas chromatography coupled to mass spectrometry (GC-MS). Alternatively, oral fluid point of care devices for on-site screening might be used but a positive result requires chromatographic confirmation.

SPE is a recommended sample preparation technique [161,162], but LLE [163] and in some cases centrifugation followed by an instrumental analysis [164] have also been reported. Several illicit drugs, such as cocaine [158,165], mephedrone [166], MDMA [167], amphetamine [152] and cannabis [150] have been detected in oral fluid. Distribution of cannabinoids have been extensively studied following cannabis smoking, which is of great importance in cases of driving under the influence of drugs (DUID). Tetrahydrocannabinol, being highly lipophilic, has been shown to deposit in the oral cavity during smoking, with concentrations of up to 1,000 ng/mL detected approximately 15 min after smoking [168]. In addition to parent drugs being detected in oral fluid, several metabolites such as BZE [169], methamphetamine [152], cannabidiol (CBD) [170] and 3,4-methylenedioxy-N-ethylamphetamine (MDEA) [167] have also been found in this matrix.

1.3.3 Head hair

Human head hair is composed of proteins (65-85%, mostly keratin), water (15-35%), lipids (1-9%) and minerals (< 1%) [171–173]. It starts growing from a follicle located 3-4 cm below the epithelial membrane, with sebaceous and apocrine glands excreting directly into the follicle [171]. Hair growth cycle is divided into three stages: anagenesis (growth phase), catagenesis (regression phase) and telogenesis (quiescence phase) [171,172]. It is assumed that head hair grows on average 1 cm per month but age, pregnancy and seasonal variations can influence the growth rate, which has been shown to vary from 0.6 cm to 3.36 cm per month [174,175].

As shown in Figure 1-19, each hair strand is composed of a cuticle, cortex and medulla [171]. An extensive network of blood vessels feed into the root of a hair, allowing substances circulating in the bloodstream to be absorbed. It takes approximately 2 weeks for a new hair to reach the scalp, and thus hair collection for drug testing should not be carried out immediately after a suspected drug intake.

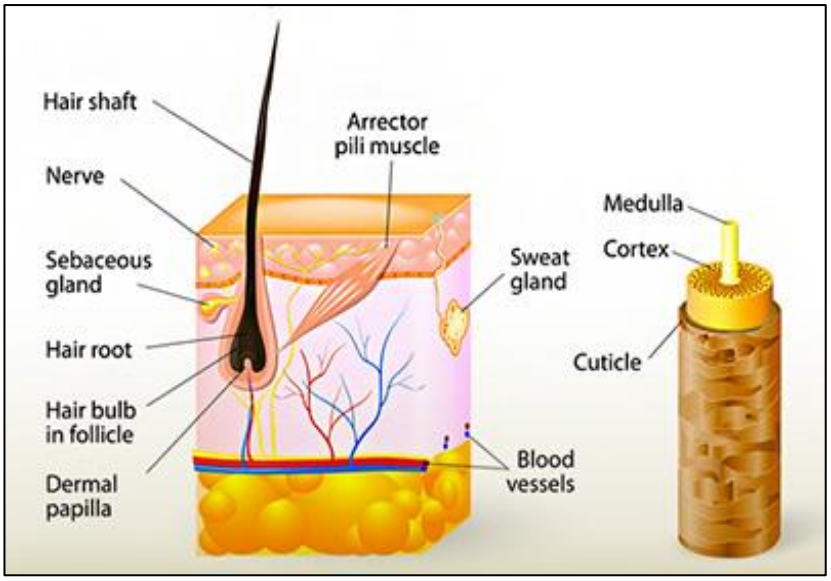


Figure 1-19. Hair structure (image taken from: http://designessentials.nl/sites/default/files/images/professional-pic-hairanatomy.jpg)

Interest in hair analysis started in 1960s when exposure to heavy metals was evaluated in head hair [176]. Nowadays, hair with its unique characteristic of allowing retrospective detection of drug use is analysed in the workplace drug testing [133,177], in cases of drug-facilitated sexual assault [178], pre-natal drug exposure [179,180], historical patterns of drug use [181], doping control in sport [182,183], alcohol abuse [184,185] or when drug administration to vulnerable groups (e.g. child custody cases) is suspected [186]. Owing to drugs being highly stable in hair, the matrix has been used to assess drug consumption in ancient North Chilean [187] and Peruvian tribes [188]. When head hair is unavailable (bald individuals or babies), pubic, arm, leg, armpit or auxiliary hair may be considered as suitable replacements. However, their growth rate is slower and more variable whereas collection of pubic hair may lead to privacy concerns [174,189].

As shown in Figure 1-20, analysis of matrices, such as blood, urine and oral fluid can provide a relatively short detection window compared to hair analysis, which can detect historical drug use weeks to months earlier [190]. The qualitative results from hair analysis are widely accepted by the scientific community but translation of detected drug concentrations in hair into an actual impairment/intoxication at the time of drug

administration still remains problematic [191]. This is further complicated in cases of single dose administration (e.g. drug-facilitated crimes), which may not result in drugs being detectable in hair [192,193].

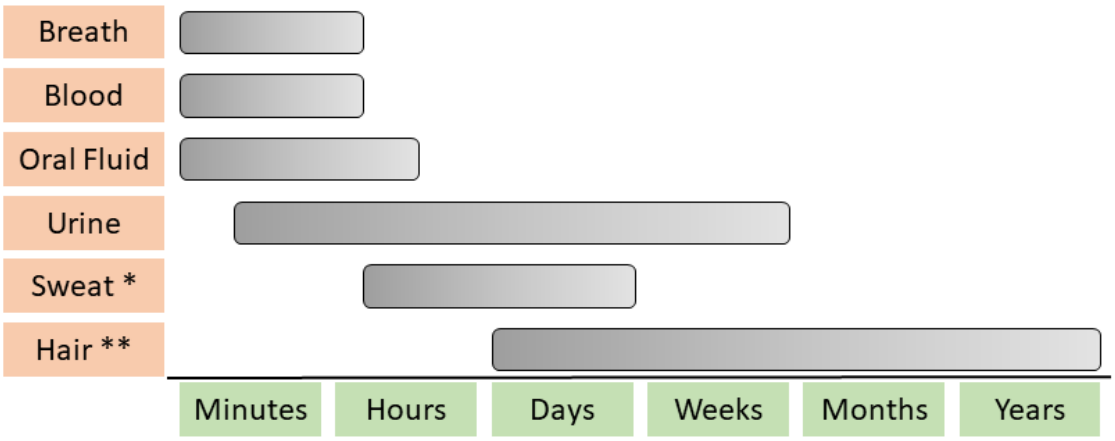


Figure 1-20. Drug detection windows for different matrices. Note that this is an estimate which is dependent on a substance, its physio-chemical properties and frequency of use; adapted from Hadland et al. [190]

** As long as sweat patchs is worn*

*** Depends on hair length*

Another reasons as to why hair has become a matrix of interest is the fact that hair does not easily decompose after death [172], offers non-invasive sample collection and ambient sample storage and transpiration [174]. However, care needs to be taken when interpreting results of hair analysis collected from children or the elderly. Lower excretion rates, more permeable BBB and varying degrees of protein binding in children as well as renal and hepatic failure in the elderly will affect blood drug concentration and, therefore, the level of impairment and drug incorporation rate into hair [194]. In addition, P450 enzymes responsible for drug metabolism mature at different rates in children [195]. CYP2D6 can take more than five years to reach adult levels while CYP3A4 matures rapidly in the first 6-12 months of life [194]. Furthermore, there are differences in the hair structure and growth. Compared to adults' hair, children's hair is more porous and its growth rate may not always follow the assumed 1 cm per month [194].

1.3.3.1 Drug incorporation into hair

Drugs can accumulate in hair growing out of follicles. As the hair located on the posterior vertex of the scalp grows 0.5 cm to 2 cm per month (often quoted as roughly 1 cm a month), the drug band in the hair closest to the scalp shows most recent drug use [174]. The exact mechanism of drug incorporation is still unknow but scientists recognise three mechanism: passive diffusion from the bloodstream during hair growth, drug excretion with sebum or sweat into the hair follicle or scalp and external contamination [171,172,174].

Physio-chemical parameters such as lipophilicity, membrane permeability and hair melanin content influence drug incorporation into hair. Favourable pH gradient between blood (pH 7.4) and the acidic hair matrix (pH 4.5-5.5) aids transportation of basic drugs into hair [192,196]. Once drugs have been trapped in the hair shaft, they will form ionic bonds with melanin and bind to lipids [197]. This may explain why cationic drugs, such as cocaine, tend to deposit more readily in hair than anionic drugs [198]. Age and sex also influence drug incorporation into hair. With age hair loses pigmentation (turns grey) and becomes thinner leading to poorer drug incorporation. In addition, female hair is usually longer and more damaged due to hair treatments.

Natural hair colour has been shown to play an important role in drug incorporation into hair. Drugs of abuse bind to a hair pigment called melanin which is present as either eumelanin (black and brown hair) or pheomelanin (red and blond hair) [171,199]. Darker hair has higher melanin content resulting in greater drug binding. Therefore, natural hair colour will have a direct influence on drug concentration in hair, with lighter hair incorporating less drug compared to darker hair. This has led to concerns of racial bias in hair testing [200]. *Rollins et al.* administered 30 mg of codeine phosphate syrup three times a day for five days followed by a 30 mg codeine 24 h after the end of the treatment to Caucasians and non-Caucasians with black, brown, blond or red hair [201]. Five weeks later codeine was found at 1,429 ± 249 pg/mg in black hair, 208 ± 17 pg/mg in brown hair, 99 ± 10 pg/mg in blond hair and 69 ± 11 pg/mg in red hair. In black Asian hair codeine concentrations were nearly three times higher than in black Caucasian hair.

According to the federal guidelines, 100% of subjects with black hair, 50% with brown hair and 0% with blond hair would have been positive for codeine. Other drugs, such as nicotine [202] and cocaine [203,204] have also been found at higher concentrations in darker hair. Seeing that drug binding to melanin leads to variable drug concentrations in different hair colours, *Hold et al.* investigated the impact of melanin removal from hair digests on cocaine quantification in the samples. Melanin removed by centrifugation did not, however, eliminate the bias [205]. Interestingly, concentrations of cannabinoids have not been found to be affected by hair pigmentation [206].

1.3.3.2 Hair treatments

Another common issue associated with hair analysis is the impact of hair treatments (bleaching, dying, perming, waving, curling and straightening), hair products (shampoo, conditioner) and exposure to UV light on drug concentrations in hair. Aggressive treatments as well as mechanical actions (brushing, banding) can damage hair and alter its porosity. Increased porosity leads to higher rates of external contaminants being incorporated into hair and compounds present in hair being washed out [207]. For example, porous hair has been shown to absorb 10-20 times more cocaine than normal hair [208]. Moreover, damaged and bleached hair has been shown to result in lower concentration of amphetamine-type-stimulants when compared to non-bleached hair [209]. Bleaching has also been reported to affect stability of opiates, benzodiazepines and cocaine in hair [189,210–212].

Washing hair with shampoo has been demonstrated to effectively remove external contaminants without extracting drugs incorporated in hair [213]. *Cirimele et al.* studied the risk of the ingredients in the cannabis shampoo being incorporated into hair following daily washing [214]. Many cannabis shampoos contain < 1% tetrahydrocannabinol (THC) but there are reports of some containing up to 3% THC. In the study three subjects washed their hair with the Cannabio® shampoo once a day for two weeks. The shampoo contained 412 ng/mL of THC, 4,079 ng/mL of cannabidiol (CBD) and 380 ng/mL of cannabinol (CBN). Hair samples were negative for THC, CBD and

CBN following extraction and analysis on GC-MS. To investigate long term effects of the shampoo, hair samples were incubated in 10 mL water:Cannabio® shampoo (20:1 v/v) for 30 min, 2 h and 5 h. The 30 min sample was negative but samples collected at 2 h and 5 h contained CBD and CBN. THC was not detected.

1.3.3.3 Hair collection and storage

There is a general consensus that hair should be collected from the back of the head, the area called posterior vertex. In this region, the rate of hair growth exhibits less variability and is less influenced by sex or age [174]. A typical protocol lists the following stages of hair collection [171,174]:

a) hair sample (usually pencil thickness) should be tied with a thread,
b) scissors should be levelled with the scalp to cut as close to the scalp as possible,
c) hair sample should be placed on an aluminium foil with the root clearly labelled and sticking out from the foil,
d) aluminium foil should then be folded and stored in a paper envelope at room temperature in a dark place.

1.3.3.4 Segmentation

The ability to detect a single dose administration is of highest importance in drug-facilitated sexual assault cases. Segmental analysis of hair is usually employed in such cases, where hair is cut into small (10 mm to 30 mm) segments. Each segment corresponds to a narrow timeframe which can help link the time of the crime with drug(s) detected in hair [215]. However, it should be noted that hair is susceptible to natural “wash-out”, which may result in drug concentration being lower in the distal end of the hair compared to the root [215].

1.3.3.5 Decontamination

Environmental contamination is a significant pitfall of hair testing. Passive exposure, where an individual is exposed to a drug (pollution, running dirty hands through hair or

smoked drugs) can often lead to false-positive results [173,216,217]. In order to minimise the chance of false-positive results, effective hair decontamination protocols are developed in testing labs, cut-off values are established and metabolites targeted in the samples [171]. Metabolites, being usually more polar then the parent drug, may not be readily detected in hair, but where possible it is recommended to use the metabolite to parent drug ratios to report positive results [173].

The first step in sample preparation is hair decontamination. Substances deposited in hair due to external sources are weakly bound to the matrix, and thus can be removed by washing with solvent(s). It is generally assumed that a complete drug removal is not achievable even after an extensive decontamination procedure. It is, however, important to use an appropriate washing solvent which will decontaminate the surface of the hair but will not extract drugs from inside the hair. The choice of solvent varies between labs, with organic solvents, water, buffers and soaps or their combinations being commonly employed [218–223]. Prolonged decontamination with some solvents, such as methanol, can make the hair shaft swell and lead to the loss of analytes from hair [224]. It has been recommended by *Tsanaclis and Wicks* to compare drug levels in washes (W) and hair (H). The W/H ratio greater than 0.5 is likely to indicate external contamination whereas the ratio smaller than 0.1 or zero would indicate drug consumption [225].

1.3.3.6 Digestion

Typically, 10-50 mg of decontaminated hair per each segment is weighed. Hair is then cut into smaller segments or pulverised in a ball-mill. Pulverised/cut hair then undergoes digestion which aims to extract analytes from the hair. Acidic or alkaline digestion, enzymatic hydrolysis or incubation with different buffers is usually used but care should be taken at this stage. Inappropriate digestion method, such as alkaline conditions, may degrade heroin, cocaine or benzodiazepines [226]. Extraction with methanol in an ultrasonic bath is time-consuming and usually takes 5-18 h [168]. Extraction with aqueous acids or buffers is well suited for basic compounds. Even though these extracts are usually cleaner than methanol extracts, partial hydrolysis of cocaine to benzoylecgonine

and 6-monoacetylmorphine to morphine is sometimes observed [218,227]. Digestion with aqueous sodium hydroxide (NaOH) at an elevated temperature is commonly used for drugs stable under alkaline conditions, such as nicotine [228,229] or amphetamines [230]. Other common methods employ the use of Proteinase K which hydrolyses hair proteins [231–233] or urea which breaks down hydrogen and disulphide bonds in hair [211].

1.3.3.7 Extraction

Digested hair contains interferences and requires sample clean-up, which usually involves LLE or SPE. The need for better sensitivity and cleaner extracts, to prevent ion suppression or enhancement on LC-MS or GC-MS, has resulted in a number of innovative extraction methods and sorbents. In headspace-solid phase microextraction (HS-SPME) lipophilic drugs of even low volatility (e.g. methadone, tricyclic antidepressants) are absorbed onto the fibre placed in the headspace above the digestion solution (usually aqueous NaOH). The retracted fibre can then be exposed again inside the GC-MS injection port. High temperature desorbs the substances from the fibre allowing them to be analysed [234–237]. The advantage of HS-SPME is automation and the solvent-free nature of sample extraction. *Strano-Rossi et al*. has applied this technique to the detection of cannabinoids in hair by immersing the fibre in the sample digest [238]. Sample clean-up by hollow-fibre liquid-phase microextraction [239] and microextraction by packed sorbent have also been reported [240].

1.3.3.8 Detection of drugs in head hair

GC-MS has been used for drug analysis for decades, showing good accuracy, specificity and limits of detection [171,174]. However, it requires sample derivatisation which makes sample pre-treatment a laborious process. Owing to the non-volatile, thermally unstable and polar nature of many drugs of abuse, LC-MS is usually a preferred choice.

In recent years, development of multi-target methods has gained pace as they offer analysis of dozen compounds in the same run, thus saving money and time. *Nielsen et*

al. have developed an ultra-high-performance liquid chromatography/time-of-flight mass spectrometry (UPLC-ToF-MS) method for the simultaneous detection of 52 common pharmaceuticals and drugs of abuse in hair. The limit of detection (LOD) in a 17 min run ranged from 0.01 ng/mg to 0.10 ng/mg [241]. More recently, *Montesano et al.* presented a UPLC-MS method for quantification of 96 drugs in 10 mg of hair [242]. The LOD ranged from 0.002 ng/mg to 0.050 ng/mg for basic drugs and from 0.10 ng/mg to 5.0 ng/mg for acidic and neutral drugs. The authors stated that low LODs may allow identification of a single dose administration. The need for high sensitivity levels in hair analysis might be further achieved by nano-electrospray ionisation which introduces mobile phase into the MS source at a flow rate of nanolitres/min. This results in the formation of smaller droplets in the ionisation process. Droplets have high surface-to-volume ratio which results in a larger proportion of ionised analytes compared to conventional electrospray ionisation (ESI) [243,244]. A nano-HPLC-Chip-MS, using a special nano-Chip-LC instrumentation was used to develop a highly sensitive method for the simultaneous detection of ketamine and norketamine in human hair [245]. Extraction of 2 mg of hair resulted in the LOD of 0.5 pg/mg for both analytes which is approximately 1000 times more sensitive than a conventional method.

Current GC-MS and LC-MS analytical methods require extensive sample preparation which is time consuming and is destructive to samples. Novel methods, such as matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) imaging and direct analysis in real time-mass spectrometry (DART-MS) aim to detect analytes in an intact hair strand or a lock of hair.

Vogliardi et al. have developed a fast screening method for the detection of cocaine in hair that was pulverised and digested. One microlitre of the reconstituted samples was deposited on the graphite MALDI holder and the sample was sprayed with alpha-cyano-4-hydroxycinnamic acid (matrix) solution. Researchers were able to achieve the LOD of 100 pg/mg [246]. MALDI-MS imaging has also been used to map the distribution of cannabinoids in a hair strand following an *in situ* derivatisation [247]. Interestingly, MALDI ionisation process has been shown to be reproducible between

individuals and not influenced by the natural hair colour (melanin content) in hair [248]. Even though MALDI-MS imaging offers quick analysis with little or no sample pre-treatment, analysis of intact single hair strands may lead to false-positive results. *Mussholf et al.* have reported that the analysis of four different hair strands from the same individual produced contradicting results, showing some samples to be negative and other samples to be positive for cocaine and its metabolites. This may be due to the growth cycle of hair strands, where 80-90% are in the growing phase while the remainder is in the regression phase [249].

Another ambient imaging ionisation technique, which does not require the addition of the MALDI matrix, is DART-MS. This relatively new ionisation technique, developed in 2005 by *Cody et al.*, uses a glow discharge inside the source which generates metastable species of a heated gas [250]. At elevated temperatures these species desorb analyte ions from the surface. DART-Orbitrap MS has been used to develop a rapid analysis of THC in human locks of hair without the need for any sample pre-treatment, reporting LODs in sub ng/mg range [251]. The use of entire locks of hair as opposed to single strands overcomes the problem of hair being in different growth cycles as described above. Even though MALDI-MS imaging and DART-MS are suitable for rapid analysis, large sample throughput and high cost of these instruments prevent widespread use.

1.3.4 Dried blood spots

Collection of dried blood spots (DBS) relies on sampling capillary blood from a finger or a heel. Capillaries, which are the smallest blood vessel in the human body, connect the smallest arteries with the smallest veins. Therefore, a sample of capillary blood is a mixture of arterial and venous blood as well as interstitial and intercellular fluid [252].

1.3.4.1 Collection devices

Capillary blood is collected by pricking a finger with a lancet. The first drop of blood, which contains tissue fluid, is discarded. Samples are then collected on a filter paper or

on a microsampling device. Before extraction/analysis, collected capillary blood is left to dry at room temperature.

1.3.4.2 Filter paper

There is a variety of filter papers used for DBS collection. Some filter papers have been specifically designed for use in drug metabolism and pharmacokinetics (DMPK) studies. DMPK-A and DMPK-B filter papers lyse cells and denature proteins on contact with blood while DMPK-C filter papers are chemical-free and are, therefore, more suitable for protein analysis [252]. Recently, non-cellulose filter paper (Agilent Bond Elut DMS) which is less influenced by the haematocrit (Hct) effect has been developed. However, it has been shown to suffer from an inconsistent saturation, fragility and poor precision attributed to the thickness of the paper [253]. More recently alginate and chitosan foam has been suggested for DBS sampling [254]. The new material gave higher recoveries than more conventional Whatman FTA DMPK and Agilent Bond Elut DMS, but the influence of the Hct was not assessed.

Spotting drops of blood onto filter paper may seem like an easy and quick collection method, but it suffers from the Hct problem which can impact precision and accuracy of an assay [252,255,256]. The ease at which a drop of blood spreads on the paper is dependent on many factors, such us donor's hydration level, type of filter paper used as well as chemical and physical properties of a drug [252,255]. The most significant factor, however, is the Hct which is defined as the percentage volume of red blood cells in a drop of blood. The higher the Hct (more red blood cells present), the more viscous the blood is. This leads to limited spreading and higher concentration of analyte(s) in the spot. On the contrary, the lower the Hct (fewer red blood cells present), the less viscous the blood. It is, therefore, prone to greater spreading yielding lower analyte(s) concentration in the spot [255] (this is illustrated in Figure 1-21). Therefore, when a fixed diameter punch-out is taken from the filter paper, punches with high Hct will contain larger volume of blood than punches from blood samples with low Hct. Furthermore, if a calibration curve is prepared in blood with a normal Hct the concentration of the samples with low Hct will

be underestimated whilst the concentration of samples with high Hct will be overestimated.

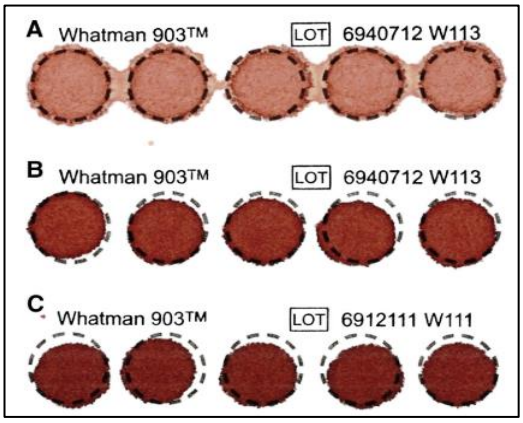


Figure 1-21. Appearance of 50 µL of human whole blood prepared at haematocrit = 0.18 (a); haematocrit = 0.35 (b); haematocrit = 0.50 (c); adapted from Wilhelm et al.[252]

Inter-individual variations in the Hct are mainly caused by age, diet and disease state. Newborns, people living in high altitudes, people suffering from polycythaemia or chronic obstructive pulmonary disease tend to have higher Hct whereas patients receiving chemotherapy or having anaemia tend to have lower Hct [257].

The Hct problem attributed to the use of filter paper has prompted scientists to propose new solutions. *Youhnovski et al.* suggested using pre-cut filter papers which would not only ensure better spreading and spot definition but would also eliminate potential carry-over caused by mechanical punching [255]. *Li et al.* used perforated filter papers, where a single use pipette tip was used to push the samples into the extraction wells [258]. Even though these methods successfully overcame the Hct problem, they required accurate sample volume to be deposited on filter paper.

Potassium has been proposed to be a useful marker of the Hct level [259–261]. Because red blood cells (RBC) are the main blood component which contains potassium, its concentration in RBC should correlate well with the Hct [262,263]. This has been shown to

be true by *Capiau et al.* who successfully predicted Hct in 3 mm DBS punches [259]. However, the authors pointed out that venous blood (preserved with lithium heparin) rather than capillary blood was used in these experiments. This might have affected their findings because heparin decreases blood viscosity [264].

1.3.4.3 Dried plasma spots

Another approach to the Hct problem focuses on the use of dried plasma spots (DPS) rather than DBS. This method has been used to detect a range of different compounds [265–269], but requires blood centrifugation and so would not be practical for home sampling.

To overcome the need for centrifugation and to eliminate the Hct problem, *Ryona et al.* developed a book-type DPS cards [270]. Capillary blood is spotted on a card, but RBC are filtered through the membrane allowing plasma samples to be collected on the layer below. This new technique has been successfully validated for quantification of opioids and stimulants in whole blood at 30%, 45% and 60% Hct levels. However, the method is not suitable for drugs with high affinity to RBC for which venous blood remains the best matrix.

1.3.4.4 Volumetric absorptive microsampling

Several methods which overcome the Hct problem have been described here, from using modified filter paper, filtering RBC to produce DPS to calculating Hct by correlating it with the concentration of potassium in blood. A different approach relies on the use of volumetric absorptive microsampling devices (VAMS; for example a Mitra® device), which accurately collect a fixed volume of capillary blood independently of its Hct [271–273]. VAMS consist of a hydrophilic polymeric tip (see Figure 1-22) and come in a clamshell (2-4 samplers) or 96-rack format. Each individual device has been designed to collect either 10 µl or 20 µl of matrix.

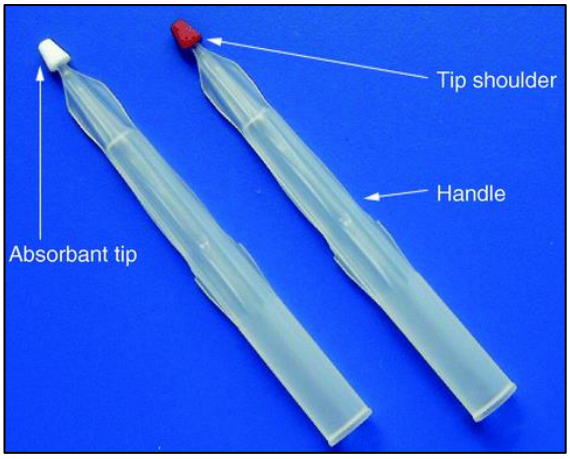


Figure 1-22. Mitra® device before (left) and after (right) absorbing capillary blood; adapted from Spooner et al. [274]

Samples are collected by wicking up capillary blood after a finger prick. The white tip on the device turns red when enough blood has been absorbed and it is usually recommended to keep it in the matrix for another 2-5 s [272,273]. Samples are then usually left for 2 h or longer at room temperature to dry. Because samples collected by VAMS do not require sample pre-treatment (centrifugation to obtain plasma or mechanical punching) they can be easily incorporated into the workflow.

VAMS have been shown to have less than 5% variation in the volume of collected blood across 20-70% Hct [272]. Similarly, a small variation has been observed when Mitra® devices were investigated in 6 different laboratories with both human and rat blood [274]. However, the difference in the minimum (9.1 µL) and maximum (13.1 µL) volume of blood taken up by the tip varied. The reasons for that are not clear but may be attributed to the differences in pipetting blood, manufacturing inconsistencies or used sampling procedure (dipping the tip too deep or not leaving it in the matrix long enough). *Mercolini et al.* have also demonstrated that Mitra® devices can be used for accurate collection of other matrices, such as urine and plasma [166]. In addition, Mitra® devices have been used for blood collection in several *in vivo* experiments after controlled drug administrations [275,276].

1.3.4.5 Application

Originally DBS were used to monitor glucose levels in rabbits [277], but more recently they have been proven useful in analysing samples from the newborns and from people collecting samples at home [278].

DBS are often chosen as a preferred matrix due to their relatively non-invasive nature and an ease of collection, which doesn't require a phlebotomist or hospital visit. From a pre-clinical point of view, the use of microsampling devices plays a significant role in the reduction of the number of rodents and the volume of matrix taken in clinical trials and toxicology studies, which fits well with the 3R (replacement, reduction and refinement) concept [258,279]. Moreover, DBS are not considered a hazardous biological material so they can be sent at room temperature by normal postal system. The fact that sample shipment does not have to be done on dry ice not only further reduces costs but can also be used in remote areas where electricity is not available [258]. Finally, DBS may offer increased analyte stability [256,275,280,281] and extended photo-stability [282]. For example cocaine has been shown to be more stable in DBS than whole blood stored for 72 h at +4°C [275]. Another study looking at incurred sample repeats analysed again after 25 months showed that lopinavir was within ± 20% of its original concentration in 14 out of 19 DBS samples whereas only 2 out of 19 plasma samples fulfilled that criterium [256].

DBS are not without their disadvantages. Because only a small sample volume is collected and very often a back-up sample is not available, sensitive and reliable analytical methods are required. For home sampling, training is usually needed although the collection process has been made easier thanks to the development of VAMS. Very often in the home setting there is an increased risk of contamination because the person collecting the sample is also the one taking prescribed medications. One of the remining concerns is the mismatch between capillary blood collected by VAMS and whole blood samples containing preservatives and anticoagulants used for the preparation of calibration standards and quality control samples. One way of mitigating this problem is the addition of the preservative onto the VAMS tip or during the extraction stage as

previously suggested by *Denniff et al* [272]. This approach has produced acceptable precision and accuracy, but a bioanalytical community is urged to discuss the problem in detail and publish an agreed solution.

1.3.4.6 Correlation of DBS drug concentrations with whole blood

Given a growing interest in DBS it is crucial to investigate drug concentration differences between capillary and whole blood. Literature suggests that this has to be done independently for each drug because drug concentrations in capillary blood may not always correlate well with whole blood [275,283,284]. This could be due to the differences between arterial and venous drug concentration. Whole blood is usually collected from a vein in a forearm whereas capillary blood obtained from a finger prick contains a mixture of arterial and venous blood. Higher arterial concentrations of fentanyl [285], nicotine [286] and diacetylmorphine [287] have been reported previously, where a shorter T_{max}, higher C_{max} and larger AUC in the arterial samples was observed. These differences are usually seen shortly after drug administration (when the drug is in the distribution phase) and become less significant once the equilibrium has been reached [287].

1.3.4.7 Detection of drugs in DBS collected on filter paper

After drying DBS collected on filter paper for 2-3 h, samples of a fixed diameter (usually 3-6 mm) are punched out and extracted using an appropriate solvent. Methanol is usually considered a good starting point for method optimisation followed by an extraction or a dilute and shoot approach [288]. Because manual extraction of DBS is a laborious and time-consuming process, fully or semi-automated methods have been reported. These can be divided into three categories: on-line desorption [289,290], paper spray [291,292] and fully automated extraction [293–297]. On-line desorption is a partly automated process where manually punched-out disks are extracted with a mobile phase or an organic solvent. The method can be further coupled up with an on-line SPE. Even though the extraction process can be sped up this way, the need for manual

punching and the need to determine the volume of spotted blood are main disadvantages. The use of paper spray for the extraction of drugs has presented itself as a promising analytical method. Paper spray, being a direct MS analysis, benefits from low sample volumes, does not require analytical columns for separation and offers quick analysis. However, samples need to be applied onto specially prepared triangular pieces of chromatography paper. Lastly, a fully automated method with barcoded DBS has been reported before but it comes at a cost. Robotic arms and handlers are quite expensive which may prevent laboratories from purchasing the equipment. It is also worth pointing out that many methods analyse DBS collected in a controlled environment whereas samples encountered in real life are likely to vary in shape, volume of deposited blood and Hct.

Drug concentration in DBS have mostly been analysed by LC-MS/MS [295,298–300], but a number of different quantitative methods, such as GC-MS [301], liquid chromatography-high accuracy mass spectrometry (LC-HRMS) [253,302], HPLC-UV [303], LC-fluorescence [304] and immunoassay [305] have also been reported. DBS have not only been applied to the quantification of small molecules but has also been shown to work with large molecules, such as hepcidin (a peptide hormone) [298] and insulin [302].

1.3.4.8 Detection of drugs in DBS collected by VAMS

VAMS tips are either removed and taken through the extraction individually or a complete 96-rack with tips is extracted as part of an automated process. The removal of tips prevents the extraction of any excess blood that might have been deposited on the handle. This problem has been reported before when a high flow of blood flowing out of rat's tail has overloaded the Mitra® device and left a visible deposit of blood on the handle [276].

Optimisation of the drying time and the selection of an appropriate extraction solvent are crucial (see Figure 1-23). In many publications samples are dried for 2-3 h at RT, however, changes in recovery and drug concentrations have been observed with the

prolonged drying step. Solvent extraction is the easiest and fastest procedure where formic acid and ammonium hydroxide are often used to improve recoveries of acidic and basic compounds, respectively. Sometimes a more time-consuming clean-up method is required, such PPT or SPE. The former is usually achieved by precipitating proteins out with methanol of acetonitrile or by using salts. VAMS manufacturers have found zinc sulfate to be the most effective salt at disrupting protein-drug binding. SPE can be further incorporated into an extraction protocol to remove interferences.

DBS collected by VAMS have been mainly analysed by LC-MS/MS [166,272,298,306–309] and HPLC-MS/MS [276,310]. Because Mitra® devices come in 96-rack format, automation is easy to achieve [298,306–308,310].

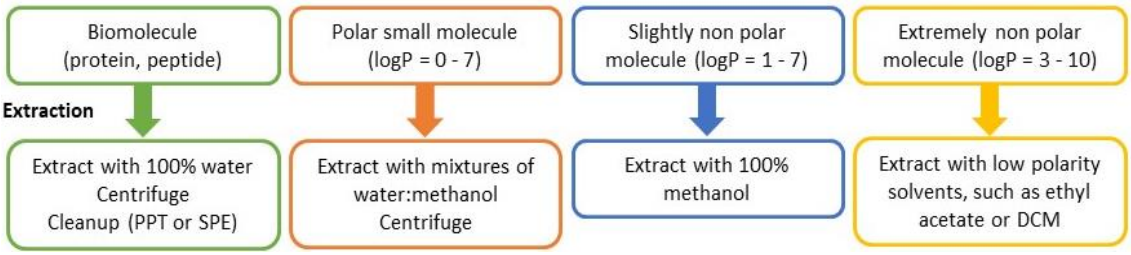


Figure 1-23. Suggested extraction protocols for small and large molecules of different polarities; acidic and basic modifiers can be added to improve recoveries.

1.3.5 Breath

Even though a wide range of analytes can be found in breath, it is less complex than blood and can be collected painlessly without the need for trained phlebotomists [311,312]. The use of breath sampling has been evaluated in prisons where inmates and members of staff preferred it to urine collection [313]. However, breath can only be used to detect very recent drug use [129]. Moreover, drugs are usually found in breath at low pg/mL level which requires sensitive detection methods [314]. Lastly, contamination of the oral cavity due to smoking or taking drugs intranasally may lead to inaccurate results.

The mechanism of drug incorporation from the bloodstream into the exhaled air is not clearly understood. Drugs need to cross an alveolar membrane in order to be found in the alveoli. The alveolar membrane is made of collagen, and thus is relatively non-polar, allowing lipophilic compounds to diffuse more effectively through the membrane. Other factors, such as molecular weight and drug-protein binding, may also impact drugs ability to reach the alveoli [312].

1.3.5.1 Exhaled breath condensate

Breath analysis is usually divided into the analysis of the volatile part and the aqueous part of breath. The latter contains non-volatile and water-soluble compounds, commonly referred to as exhaled breath condensate (EBC). Collection of EBC is relatively easy as it requires breath to flow down a cooled tube. At low temperatures, compounds present in breath condensate and are collected [315]. A number of portable and highly advanced benchtop EBC collection devices have been developed and applied in the field of toxicology. However, a significant limitation of the EBC analysis is the lack of standardisation. Moreover, because EBC consists of 99.9% water, compounds found in EBC are highly diluted, which not only calls for sensitive instrumentation but also leads to high inter- and intra-subject variability [315].

1.3.5.2 Exhaled particles

In addition to EBC, a new method for capturing microscopic particles from breath has been developed by a Swedish company, Pexa. The company use a stationary collection system which captures particles, separates them according to size and allows each fraction to be analysed by time-of-flight secondary ion mass spectrometry (ToF-SIMS). Thanks to this method researchers were able to characterise phospholipid composition in the exhaled particles from patients with asthma and cystic fibrosis [316].

1.3.5.3 Particle filter devices

Another way of breath collection uses a particle filter device which relies on analytes exhaled in breath being trapped on a polymeric filter. A commercial design of a particle filter device has been released by SensAbuse®. The device was used to detect a panel of 12 illicit drugs in breath samples collected from 47 patients [317]. All drugs were detected in breath samples, but low levels of detection were observed for benzodiazepines. *Stephenson et al.* have recently published a fully validated routine screening method for quantification of 9 drugs and have successfully applied it to the analysis of 1,096 breath samples. Positive findings were reported in 3.6% of all cases, with amphetamine (25 cases), methamphetamine (10 cases) and THC (8 cases) being the most frequently detected [314].

1.3.5.4 Application

One of the well-established areas where breath testing is widely used and accepted is the measurement of blood alcohol level in impaired drivers [190]. Exhaled breath contains over 3,500 endogenous compounds, many of which characterise the function of the respiratory system [318]. At the moment only a handful number of diseases can be effectively diagnosed by breath analysis, such as diabetes [312,319] or an infection with *Helicobacter pylori* [320]. In addition, biomarkers of lung diseases (e.g. asthma, lung cancer, chronic obstructive pulmonary disease) are likely to be found in breath. Several compounds have already been studied as potential biomarkers, but the search for the most effective biomarker(s) is still ongoing [315,318,321].

1.3.5.5 Detection of drugs in breath

First attempts of drug detection in exhaled breath started in early 1980s. GC-MS was initially used to detect a range of volatile small molecules, such as ethanol, THC and γ-butyrolactone [322–324]. More recently amphetamine and methamphetamine [325] as well as proteins, peptides, lipids and other endogenous compounds have been detected in

human breath by LC-MS/MS [315,318]. Other techniques, such as ion mobility spectroscopy [326], selected ion flow tube mass spectrometry [327], surface-enhanced Raman spectroscopy [328] and electronic noses [329] have also been used for detection of volatile compounds in breath.

The detection of fentanyl in breath samples from six patients who received intravenous fentanyl was firstly reported by Wang *et al* [330]. Breath samples were collected from the expiratory circuits of an anaesthetic machine and were analysed by GC-MS after solid-phase microextraction. *Takita et al.* used proton transfer reaction mass spectrometer to measure propofol concentrations in plasma and breath. Good agreement between propofol concentrations in both matrices was reported [331]. Methadone has been detected in EBC collected from patients undergoing methadone maintenance treatment [332]. Methadone excretion in breath ranged from 0.390 ng/min to 78 ng/min but the study did not aim to detect the metabolite of methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP). The presence of EDDP in breath was confirmed in a different study which collected samples from patients on the methadone substitution programme [333]. Metabolites of other illicit drugs have also been detected in breath. CBN, which is a metabolite of THC, was found in breath samples collected from a controlled administration study where chronic and occasional cannabis smokers were given cannabis cigarettes containing 6.8% THC (54 mg) [334]. Moreover, *Beck et al.* detected 6-monoacetylmorphine at concentrations ranging from 42 pg/sample to 6,080 pg/sample in 6 breath samples collected from 47 patients undergoing recovery from acute drug intoxication [317].

1.3.6 Sweat

Sweat is an important biological matrix responsible for maintaining homeostasis by keeping bodily temperature in a narrow range. Sweat is mostly composed of water (approximately 99%) [335] as well as inorganic salts, fatty acids, urea, ions, amino acids and a range of other compounds excreted through the skin or picked up from the

environment. The wide range of compounds present in sweat illustrates its complexity and a potential for inter-subject variability [129].

As shown in Figure 1-24, there are two types of sweat glands: eccrine and apocrine. Eccrine glands are distributed almost all over the human body and play an important role in cooling by performing water-based secretion. Apocrine glands secret concentrated fatty sweat and are found in certain places on the human body, such as armpits, scalp and genital region.

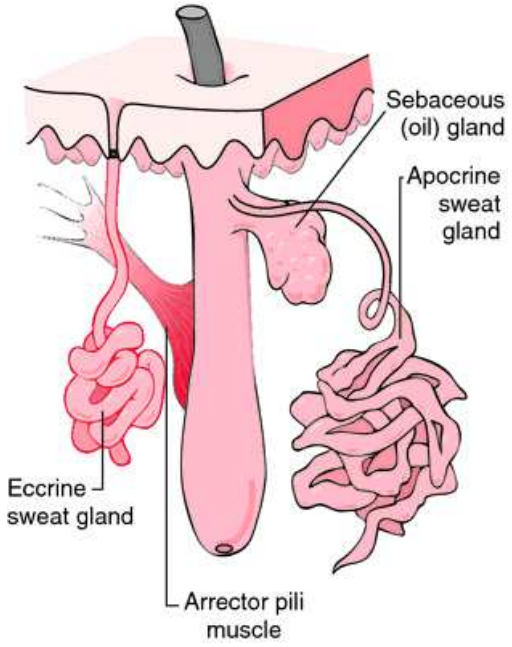


Figure 1-24. Schematic representation of sweat glands (image taken from: http://medical-dictionary.thefreedictionary.com/gland)

Collection of sweat is non-invasive [300,336] and marked with high subject acceptability [337]. Compared to urine, sweat offers a longer drug detection window and is more difficult to adulterate [336]. Sweat patches allow drug intake over several weeks to be monitored [337] and cumulative exposure to be measured [133]. However, external contamination remains a problem. Several court cases from individuals under supervised pre-trial or probationary release have been dismissed following positive sweat patch results for methamphetamine, contradicting individuals' claims of being drug free. Urine tests

came back negative and so false positive nature of the patches was concluded to be a result of environmental contamination [338]. Moreover, the use of patches suffers from high inter-subject variability, accidental or deliberate removal and difficulties with data interpretation in cases of lipophilic drugs which tend to be excreted over time. Lastly, the need for sensitive analytical instrumentation is vital [337].

1.3.6.1 Drug incorporation into sweat

The exact mechanism describing the incorporation of drugs into sweat is not well understood but the process is regulated by the physico-chemical properties (protein binding, molecular weight, pKa, lipophilicity) [339]. Lipophilic drugs cross membrane barriers more easily than their hydrophilic metabolites or polar drugs, and thus will be found in sweat at higher concentrations [340]. Basic drugs will concentrate in fluids of lower pH than that of blood/plasma (mean pH: 7.4) [339]. Sweat has a slightly acidic pH (mean pH: 6.3) which means that basic drugs will become ionised in sweat.

It has been postulated that the concentration gradient allows for passive diffusion of the unbound drugs from the bloodstream into sweat glands through the lipid bilayer [335]. Another proposed mechanism of drug incorporation is a passive diffusion through the dermal and epidermal layers in the skin as well as excretion of substances via sebum [336,337,340].

1.3.6.2 Collection devices

Stimulation of sweating by electrical diffusion of pilocarpine into the skin [341] or by warming the skin (wrapping/gloves) [157] have been reported before but is not practical for drug testing/remote sampling.

Sweat patches are most commonly used to collect sweat over days/weeks. PharmChek® is an example of the most commonly used sweat patch [336]. The patch consists of an absorptive cellulose layer covered with a semi-permeable membrane which is usually

worn for a week when approximately 2 mL of sweat is absorbed onto the patch. Drugs are trapped onto the absorptive pad while water vapour and other gases pass through it. The patch has been used in a number of studies, including controlled drug administrations [336,342] and patient monitoring [300]. One of the reported drawbacks associated with the use of sweat patches is the problem of drugs being reabsorbed into the skin (from the patch) which may contribute to the inter-subject variability [343].

Another method of collecting sweat is with wipes/swabs. A commercially available immunochemical device called DrugWipe® has been proven useful in cases of DUID [344] and in PK studies [345]. Manufacturers reported fast detection (5 min) of up to seven drug groups. Furthermore, reports of using cotton swabs soaked in alcohol to collect sweat from the forehead of cocaine users' children have also been published [346].

Due to their non-invasive and easy sample collection, the use of fingerprints for drug testing has recently become a centre of focus. Deposited fingerprints carry a unique additional benefit of person identification based on the finger ridge detail. When a finger touches a surface, eccrine sweat, external contaminants and sebum leave an invisible ('latent') fingerprint pattern [347]. Fingerprints can be collected simply on a piece of paper or glass. An alternative method suggested by *Archer et al.* involves a grooming procedure to mimic the natural composition of a sample as well as to minimise sample variability [348]. The grooming procedure involves cleaning hands with an ethanol wipe and then using both hands to wipe specific areas of the face in a repetitive manner (10 strokes/times), passing hands through hair (10 times) and finally rubbing fingertips together for a few seconds.

1.3.6.3 Detection of drugs in sweat

A number of studies have previously reported the detection of illicit drugs (either parent or Phase I metabolites) in sweat [157,349]. Phase II metabolites have also been detected in fingerprint sweat before [350]. GC-MS [336,342,343,345,351–354] and LC-MS/MS [300,350,355] have been applied extensively to the detection and quantification of drugs in sweat patches,

swabs and fingerprint sweat. *Jacob et al.* have detected methadone and EDDP in two single fingerprints collected on glass cover slips from eight patients on a methadone maintenance programme. Interestingly, the study also reported varying concentrations of methadone on deposited fingerprints from 3 different fingers. Authors suggested that the surface area of the fingertips and the amount of collected sebum and sweat may be a potential explanation [355]. Moreover, the first study which reported the presence of a Phase II glucuronide metabolite in ten combined fingerprint deposits was published by *Goucher et al.* The detection of a Phase II metabolite in a sample is important from a forensic point of view because it indicates the drug has been metabolised and, therefore, excludes the possibility of environmental contamination [350].

In addition to LC-MS/MS and GC-MS several other approaches have been reported for the detection of drugs in fingerprint sweat. Antibody-nanoparticles conjugates have been proven useful in the detection of drug metabolites in sweat deposited in latent fingerprints [356–358]. Ambient imaging techniques, such as surface-assisted laser desorption/ionisation-mass spectrometry (SALDI-MS) [359], desorption electrospray ionisation-mass spectrometry (DESI-MS) [360–362], paper spray-mass spectrometry [363] and MALDI-MS imaging [362,364] have also been utilised. These imaging techniques can not only visualise the ridge detail but can also show the distribution of endogenous and exogenous components of sweat, which has been demonstrated in fingerprints from drug users [359,365] and in dusted fingerprints [359,361]. However, challenges related to analytical sensitivity and large inter-subject variability in the state of collected fingerprints still remain [364].

1.4 Importance of NPS detection in clinical and forensic drug testing settings

Despite analytical challenges associated with detection of NPS (see 1.1.3 for more details), it is important to target new emerging drugs (including mephedrone) in clinical and forensic drug testing settings.

1.4.1 Clinical setting

When biological samples are taken for drug testing from patients suffering from acute drug toxicity, results are usually not received in time to inform clinical management of patients, who remain in hospital on average 4-5 h [366]. In addition, most hospitals do not have analytical technologies or methods capable of identifying NPS, especially the emerging ones. Nevertheless, identification of NPS via analytical screening is vital in informing clinicians about new patterns of toxicity, providing information to the legislative bodies about the risks associated with new substances and confirming the presence of NPS for when case reports or case series are published [13].

1.4.2 Forensic setting

In the forensic context, detection and identification of NPS is crucial in driving under the influence of drugs (DUID), drug facilitated sexual assault (DFSA), post-mortem analysis and in the workplace drug testing.

Studies investigating the link between driving and synthetic cathinones are scarce. However, adverse effects associated with the use of synthetic cathinones (palpitations, dizziness, light-headedness, paranoia; see 1.2.9.1 for more detail) can have a major impact on driving ability [367]. Blood cut-off concentrations in DUID cases have not been defined for NPS, but there are reported cases of individuals driving under the influence of NPS, including mephedrone [70,368].

Another aspect of forensic toxicology where drug detection capabilities are crucial is DFSA. NPS are not usually associated with DFSA, but a recent publication identified methylone (synthetic cathinone) in 13% of 45 sexual assault samples analysed by the University of Miami Forensic Toxicology Laboratory [369]. The authors hypothesised that methylone was used because it is capable of producing a mental state of confusion, but they also highlighted the need for monitoring new patterns of drug use.

Even though it can be difficult or even impossible to attribute post-mortem concentrations of NPS to recreational or excessive use, determination of the cause of death is equally important in forensic toxicology. Deaths caused by sole mephedrone use are rare (see 1.2.9.4 for more details related to mephedrone fatalities) but in 2018 there were 125 deaths involving NPS, a significant increase from 61 deaths recorded in 2017 in the UK [370].

The use of drugs in the workplace is a growing problem that poses a potential health and safety risk to the drug users, their fellow colleagues and clients [371]. The addition of the NPS (including mephedrone) to the library of drugs screened for in the workplace testing is, therefore, important because NPS can cause acute toxic effects and impaired judgement, presenting a significant risk to safety in situations where industrial machinery is operated, dangerous chemicals are handled or when driving motorised vehicles. Cut-off concentrations for NPS in urine, oral fluid or head hair have not been defined by the international bodies, such as the European Workplace Drug Testing Society. Therefore, laboratories tend to decide on the scope of substances included in a routine screen and adopt their own cut-off concentrations, which may vary widely between drug testing providers [13].

1.4.3 Detection of mephedrone and its metabolites in alternative biological matrices

Alternative biological matrices are being increasingly evaluated for use in clinical and forensic drug testing settings, but there is little available research investigating the viability of these biological matrices for detection of NPS, including mephedrone and its metabolites. This is illustrated in Table 1-2, which shows that mephedrone metabolites have not been widely targeted in alternative biological matrices. Mephedrone itself was only quantified by LC-MS/MS in authentic oral fluid, DBS and head hair samples.

In oral fluid, several analytical methods for quantification of mephedrone by GC-MS [372,373] or LC-MS [166,374,375] have been developed, but only two of them analysed real

samples. *Strano-Rossi et al.* published a LC-MS/MS screening method for the detection of NPS in oral fluid [374]. When the method was applied to 400 real oral fluid samples from traffic control stops, mephedrone was not detected. In the other study, *Mercolini et al.* detected mephedrone at 38 ng/mL and 15 ng/mL in unstimulated oral fluid collected by a disposable plastic pipette from self-reported drug users [166].

To date the only publication which reported a successfully validated method for the detection of 64 NPS (including mephedrone) in DBS (collected on filter paper) was published by *Ambach et al.* The method was applied to 21 authentic capillary blood samples but mephedrone was not detected [376].

Mephedrone has been targeted in hair samples collected from suspected drug users and analysed as part of forensic casework [116,377–381]. Quantitative results were only presented in some of these studies. Following LC-MS analysis, *Salomone et al.* detected mephedrone in two samples from proven MDMA and/or ketamine users at 50 pg/mg and 59 pg/mg [380]. *Martin et al.* found mephedrone at higher concentrations of 26.8 ng/mg (range: 0.2-313.2 ng/mg) in hair samples submitted to the ChemTox Laboratory in France [381]. Post-mortem hair concentrations of mephedrone were reported by *Torrance et al.* who detected the drug at concentrations between 4.2 ng/mg and 4.7 ng/mg following GC-MS analysis [116]. The only method which targeted mephedrone metabolites (nor-mephedrone and dihydro-nor-mephedrone) by LC-MS did not detect them in the hair samples from 154 healthy volunteers [378].

Table 1-2. Summary of the studies reporting detection of mephedrone and its metabolites in the alternative biological matrices; NR – not reported

Matrix	Number of subjects	Analytical method	Mephedrone detected?	Metabolites targeted?	Metabolites detected?	Ref
Oral fluid	n = 5	LC-MS/MS	✓	×	-	166
	n = 400	LC-MS/MS	×	×	-	382
Head hair	n = 67	GC-MS	✓	×	-	381
	n = 1	GC-MS	✓	×	-	116
	n = 16	LC-MS/MS	×	×	-	377
	n = 154	LC-MS/MS	✓	✓	×	378
	n = 325	LC-MS/MS	✓	×	-	379
	n = 77	LC-MS/MS	✓	×	-	380
DBS	n = 21	LC-MS/MS	×	×	-	376
Breath	NR					
Sweat	NR					

1.5 Aims and objectives

The aim of this study was to conduct an ethically approved controlled single dose administration of nasally insufflated mephedrone to investigate the distribution of mephedrone and its major Phase I metabolites in head hair, oral fluid, sweat, DBS, breath, urine, plasma and whole blood. The secondary aim was to report for the first-time pharmacokinetic and pharmacodynamic information associated with mephedrone and its metabolites after nasal insufflation. Although a pilot study and the first reported controlled human mephedrone administration studies have been published (see 1.2.8 for more details), they have certain limitations that our research seeks to address. Firstly, by conducting a human administration study with mephedrone purchased from a certified commercial supplier, the exact dose and purity of the drug given to the participants will be known. This was not the case in the pilot study where the research team obtained mephedrone from police seizures while in the controlled

administration study mephedrone was obtained from the Spanish Ministry of Justice and the Ministry of Health. In both cases no information on drug purity was provided. Furthermore, there is limited data available on human oral bioavailability of mephedrone, thus it is difficult to determine how oral doses in the aforementioned studies compare to doses administered by the intranasal route used in our study. Finally, both research groups only collected conventional samples (plasma and urine) from the participants, whereas as part of this study several alternative biological matrices will be collected and analysed for mephedrone and its metabolites. It is hoped that the data gleaned from this PhD project will be of value in forensic (drug-related deaths and crime) and clinical (acute drug toxicity and drug dependence) toxicology as well as in the workplace and roadside drug testing.

CHAPTER 2

MEPHEDRONE ADMINISTRATION STUDY, ANALYTICAL METHOD DEVELOPMENT AND VALIDATION

2.1 Design of the mephedrone administration study

A controlled human drug administration study is the most insightful way of understanding drug metabolism, distribution and pharmacokinetics. Not only does it allow clinicians to control the dose and purity of a given drug, but it also ensures no poly drug use at the time of administration. This is crucial to fully understand the sole action of a drug which might be altered through CYP2D6 liver enzymes being induced or inhibited when taken with other illicit or prescribed substances. Moreover, the exact time of drug administration is known which is often not reported in clinical cases involving patients presenting to the Accident and Emergency Department. Furthermore, some patients suffering from acute drug toxicity may have problems remembering the name of the drug taken. Some drugs have similar names, such as mephedrone, methadone or methedrone, making it difficult for the patients to give an accurate description of the drug.

Gamma-hydroxybutyrate [383], cocaine [284] and diazepam [350] are just few examples of drugs which have been administered to healthy volunteers in a controlled setting before. Controlled mephedrone administration studies reported by *Olesti et al.* [91,97] and *Papaseit et al.* [96,384], where doses of up to 200 mg were given orally to healthy male volunteers, formed the basis of the safety assessment of our study and helped to justify safe dosing.

Full ethical approval for the controlled mephedrone administration study was obtained from the Riverside National Research Ethics Service (16/LO/1342) and the approval letter can be found in Appendix A.

2.1.1 Study advertisement

The study was advertised on the King's College London research recruitment website and in two local newspapers: Evening Standard and Metro.

2.1.2 Inclusion and exclusion criteria

Participants enrolled into the study were non-smokers between 18 and 40 years old. They were occasional users of mephedrone or other stimulant drugs but were drug-free 2 weeks before the study day (i.e. mephedrone administration) and 30 days after the study day. Participants had no underlying hypertension (> 140/90 mmHg) and were not suffering from chronic cardiac/respiratory/renal diseases. In addition, participants were not taking over-the-counter/prescribed drugs and/or herbal/alternative remedies at the time of the study. Furthermore, those with a history of closed angle glaucoma or involved in another clinical trial were excluded. Female volunteers could not take part in the study due to the unknown effects of mephedrone in undetected pregnancy.

Participants were asked not to cut, dye, perm or bleach their head hair over the period of 30 days from the day of mephedrone administration. These requirements were in place because hair treatments can affect drug concentration in hair. Additionally, only participants with hair length of more than 1.25 cm, which allowed for the collection of an adequate amount of hair for analysis, were accepted into the study.

2.1.3 Study visits

Participants were required to attend 6 study visits:

- Screening visit 30 days before mephedrone administration
- Urine collection 1 week before mephedrone administration
- Mephedrone administration (referred to here as a study day)
- Day 2, Day 3 or 4 and Day 30 post mephedrone administration

Screening visits and drug administration took place in the Clinical Research Facility (CRF) in St Thomas' Hospital where cardiovascular monitoring and biological sampling was undertaken. Urine collection and post mephedrone administration visits took place at King's College London.

2.1.4 Screening visit

At the screening visit potential participants had the opportunity to discuss the study with a specialist registrar. Participants also underwent a physical examination, 12-lead electrocardiogram (ECG), baseline observations (blood pressure, pulse, blood oxygen saturation, height, body weight and body temperature), and were asked about their medical history (including inclusion and exclusion criteria as well as history regarding current and previous recreational drug use). In addition, participants were asked not to take recreational drugs/NPS from 2 weeks prior to the study day to 30 days after it. This was to ensure that participants were mephedrone/metabolite free and, therefore, metabolites detected on the administration day were the result of administered mephedrone. From a safety perspective it was also crucial to eliminate the risk of drug-drug interaction due to poly drug use.

2.1.5 Urine collection

Seven days before the mephedrone administration day, a urine sample was collected from each participant and analysed using a standard stimulant (including mephedrone)

screen at Abbott (previously Alere Toxicology). This was done to ensure drug-free compliance. If a positive result was reported, the participant was removed from the study. If a negative result was reported, the participant was allowed to proceed with the study.

2.1.6 Mephedrone administration day

On the main study day there was a minimum of five research staff present in the CRF. At all times participants were attended by two doctors of at least registrar/specialist registrar grade and a consultant member of the Clinical Toxicology team was in the hospital available for advice or to attend if necessary. Furthermore, the CRF had a full resuscitation equipment if required and was supported by the St Thomas' Hospital cardiac arrest team.

Prior to the mephedrone administration (i.e. at -10 min) each participant had their baseline observations (heart rate, blood pressure, body temperature, blood oxygen saturations) checked. If participants fulfilled the criteria, baseline biological samples were taken. Participants were then asked to wear a disposable gown over their clothes, a pair of gloves and a hair cap while they self-administered a single 100 mg dose of mephedrone powder via nasal insufflation using a straw. This was done in a different room to avoid contamination and was overseen by someone not involved in the other aspects of the study. Participants were then asked to clean their hands and face with ethanol wipes and were taken back to the main study room where biological samples were collected at varying times over the next 6 h (Table 2-1).

Participants had their cardiac rhythm continually monitored and recorded at each point of sampling or every 20 min (whichever was shorter). To determine whether participants were experiencing adverse neuropsychiatric effects, participants were regularly asked a series of questions using a validated score incorporating a visual analogue scale (VAS) [385,386]. This was done at -10 min and then every 15 min from the time of mephedrone administration until 2 h and then at 2.5 h, 3 h, 5 h and 6 h. The test assessed subjective

experiences of: “drug effect, bad effect, good drug effect, high, stimulated, sad, confused, fearful, liking, dizzy, experiencing changes in distance, light, hearing, body sensation, surrounding”. VAS was presented as 10 cm horizontal lines, where the 0 cm point indicated no effects/changes and the 10 cm point indicated extreme effects/changes.

If at any point the heart rate exceeded 140 bpm, blood pressure exceeded 180/100 mmHg, temperature exceeded 38.0°C or if the participant became agitated or developed adverse effects that were related to mephedrone and considered significant by the attending Clinical Toxicologist, the study was stopped. At this point no further biological samples were taken, continuous physiological monitoring continued, a 12-lead ECG performed, and the participant was cared for by the attending Clinical Toxicology team. If additional medical treatment(s) were required to manage the heart rate, blood pressure and/or temperature a consultant Clinical Toxicologist determined whether admission overnight for ongoing observation was required.

Table 2-1. A detailed outline of the frequency and sample types taken after mephedrone administration

** sample collection at indicated time ± 2 days*

Time	Whole blood	Urine	Hair	Oral fluid	Fingerprint sweat	Head sweat	DBS	Breath
-10 min	x	x	x	x	x	x	x	x
100 mg mephedrone hydrochloride administrated by nasal insufflation								
5 min	x			x			x	
10 min	x				x			x
15 min	x			x				
20 min	x				x		x	
30 min	x			x				
45 min	x				x	x		x
60 min	x			x			x	
75 min	x							x
90 min	x			x	x	x		
105 min	x						x	x
2 h	x			x			x	
2.5 h	x			x				
3 h	x				x	x		x
5 h	x			x	x	x		
6 h	x	x					x	x
Day 2	x	x		x	x		x	x
Day 3 or Day 4	x	x		x	x		x	x
Day 30 *		x	x			x		

2.1.7 Follow-up visits

Participants were asked to come to King's College London for the follow-up visits on Day 2, Day 3 or 4 and Day 30 post mephedrone administration to enable the collection of biological samples (see Table 2-1) and to ensure participants' well-being.

2.1.8 Source of mephedrone powder

Mephedrone hydrochloride powder was purchased from Chiron (Trondheim, Norway), who provided a certificate of authenticity and certificate of origin stating the purity of the drug. As an additional precaution, purchased mephedrone was further analysed in-house by LC-MS and proton nuclear magnetic resonance (H^1 NMR) to ascertain structure authenticity and to ensure it met acceptable purity.

Mephedrone hydrochloride was stored under the Schedule 1 licence held by the Drug Control Centre (DCC) at King's College London. It was weighted under the supervision of a senior scientist and transported to St Thomas' Hospital on the day of mephedrone administration where it was put in a safe in the CRF until required.

2.1.9 Justification for the mephedrone dose

The initial dose of mephedrone taken by recreational mephedrone users ranges between 15 mg and more than 300 mg for oral ingestion and between 5 mg and 250 mg for nasal insufflation [66]. Because of mephedrone's short duration of action, recreational users commonly re-dose a number of times in a single session such that they use up to 1 g or more per session [68]. Case reports and series of acute mephedrone toxicity have generally reported use of 300 mg to 7,000 mg [33,69]. Moreover, in a dose-finding pilot study and recent human administration studies, 50-200 mg of mephedrone given orally were well tolerated [95,384]. Therefore, the dose of 100 mg used in this study represented a safe dose for the participants and it was also a dose that ensured that the

concentrations of mephedrone and its metabolites as well as the pharmacokinetic data were relevant to both clinical and forensic cases.

2.1.10 Chosen route of administration

This is the first controlled human administration study where mephedrone was given via nasal insufflation, which is the most common route of administration by the recreational mephedrone users [33]. In other studies, where healthy male volunteers were given mephedrone, the drug was given orally [91,95,97].

2.1.11 Sample collection

The collection times outlined in Table 2-1 were decided based on the information from the recreational mephedrone users which suggested that intranasal administration is associated with an onset of action of 2-10 min and a peak action within 30 min [33]. This was the only information available at the time of writing the ethical approval in late 2015. Detailed description of each sample type and the collection process is described below.

- **Whole blood and plasma**

To allow for multiple blood sampling without the need for repeated venepuncture, participants had an intravenous cannula inserted into their forearm. At each timepoint, a syringe was used to withdraw and discard 2 mL of blood which is a mixture of both saline and blood. A 5 mL blood sample was then collected into a vacutainer containing sodium fluoride/potassium oxalate (NaF/KOx; 12.5 mg/10 mg) preservative and the intravenous line was flushed with 5 mL of 0.9% sodium chloride solution. At each timepoint two aliquots of blood were collected, one of which was centrifuged at 2300 rpm for 10 min to harvest plasma. Remaining blood samples collected on Day 2 to Day 30 were taken by a trained phlebotomist using venepuncture of a forearm vein. Plasma and whole blood samples were stored at -20°C and +4°C, respectively, until analysis.

❖ **Urine**

Urine samples were collected at time intervals outlined in Table 2-1 with extra samples being collected between the administration and 6 h if a participant felt the need to pass urine. The volume of excreted urine was recorded. Urine was collected into Nalgene® storage containers and stored at -20°C until analysis.

❖ **Head hair**

Hair samples (7 mm in diameter) were collected before mephedrone administration and 30 days after. Hair was cut as close to the scalp from the posterior vertex as possible (leaving approximately less than 0.25 mm *in situ*). Following hair collection, the proximal end of the samples was wrapped in a piece of tinfoil with the root end clearly marked. Hair samples were stored desiccated in envelopes at room temperature.

❖ **Oral fluid**

Oral fluid was collected by a Certus® collection device provided by Abbott (previously Alere Toxicology). Participants were asked to keep a swab under the tongue until the blue dye appeared, which indicated sufficient sample volume. The swab was then transferred to a buffer solution which was shaken for 30 s. The swab was removed from the solution and buffered oral fluid sample was stored at -20°C until analysis.

❖ **Dried blood spots**

A finger was cleaned with an ethanol wipe and allowed to dry. A single-use lancet was then used to prick the finger and capillary blood was collected directly onto the filter paper and onto a Mitra® collection device. Filter papers and Mitra® devices were dried for 2 h at room temperature and were stored desiccated at -20°C until analysis. The forefinger was usually used for sample collection but sometimes participants reported the pricked finger getting swollen causing them discomfort. In those cases, another finger was used for sample collection.

❖ **Fingerprint sweat**

At -10 min and 10 min, fingertips were cleaned with an ethanol wipe and allowed to dry. A print from each finger of the hand that was not used for DBS collection was deposited onto the surface of a clean circular glass cover slip (two samples were collected at 90 min, 3 h and 5 h). In parallel to the glass cover slips, fingerprint sweat was also collected onto triangular pieces of chromatography paper placed on top of a scale. Participants were asked to push down on the piece of paper for 10 s to give pressure between 800-1200 g. Glass cover slips were transferred into scintillation vials whereas triangular pieces of chromatography paper where kept desiccated in a box. All samples were stored at -20°C until analysis.

❖ **Head sweat**

Sweat deposits were collected by wiping the surface of the forehead 5 times with a cotton swab. Swabs were then placed in glass vials and stored at -20°C until analysis.

❖ **Breath**

A DrugTrap® device provided by SensAbues was used to collect breath. The device separates saliva and larger particles from the micro-particles of interest which are collected on a polymer filter inside the device. The sampling procedure was standardised by filling a plastic bag with approximately 20 L of exhaled breath after blowing for 1 min. DrugTrap® collection devices were sealed with plugs and stored at -20°C until analysis.

❖ **Buccal swab**

At the beginning of the study two buccal samples were taken, one from each cheek. The inside of the cheek was gently rubbed with a sterile cotton swab for 10 s. Samples were placed in a tube and stored at +4°C until analysis.

2.1.12 Sample analysis

Collected samples were transported from the CRF to the following destinations using Human Tissue Act (HTA) approved procedures:

- whole blood, plasma, urine, fingerprint sweat (collected onto glass cover slips), DBS (collected by the Mitra® device) and head hair samples were analysed using a validated LC-MS/MS method at King's College London, UK
- oral fluid was analysed using a validated LC-MS/MS method at Abbott (previously Alere Toxicology), UK
- DBS (collected on the filter paper) were analysed at Ghent University, Belgium
- aliquot of plasma samples was sent for the metabolomics analysis to the University of Zurich, Switzerland
- extra fingerprint sweat samples collected onto glass cover slips at 90 min, 3 h and 5 h were analysed via DESI-MS by Waters Corporation, UK
- fingerprint sweat samples collected on the triangular pieces of chromatography paper were analysed via paper spray-MS at the University of Surrey, UK
- single strands of hair were analysed by MALDI-MS imaging at Sheffield Hallam University, UK
- buccal swabs were analysed by the Massive Parallel Sequencing technology at King's College London, UK

2.1.13 Recruitment outcome

Seven healthy male volunteers were recruited for the study. Six of them completed the study and one was not able to take mephedrone because of high blood pressure on the mephedrone administration day. All participants were compensated with London living wage for the time they participated in the study. Physical characteristics of the participants are presented in Table 2-2.

Table 2-2. Physical characteristics of the participants who completed the study

** body mass index*

Participant	**Dose of mephedrone taken** (mg)	**Weight** (kg)	**Height** (cm)	**BMI ***
M1	99.7	84.0	184	24.8 (healthy)
M2	100	76.4	177	24.2 (healthy)
M3	102	66.3	170	22.9 (healthy)
M4	99.4	59.0	168	20.9 (healthy)
M5	99.4	82.2	196	21.3 (healthy)
M6	99.8	55.2	164	20.5 (healthy)

2.2 Statistical analysis

Statistical analysis was performed in Microsoft Excel 2016 or in GraphPad Prism software (version 7.0).

Standard deviation (SD) and the coefficient of variation (%CV) were calculated using Equation 2-1 and Equation 2-2, respectively.

Equation 2-1. Equation for calculating standard deviation where $\bar{x}$ is a sample mean and N is a number of observations.

$$SD = \sqrt{\frac{\Sigma(x - \bar{x})^2}{N - 1}}$$

Equation 2-2. Equation for calculating the coefficient of variation (%CV)

$$\%CV = \frac{SD}{mean} * 100\%$$

The Pearson correlation coefficient was calculated according to Equation 2-3. One sample t-test (t, p-value < 0.05) was calculated using Equation 2-4. If the calculated t-

value fell outside the range defined by the critical value from the 2-tailed t-Table, the t-value was considered to be statistically significant. Both tests were performed in GraphPad Prism (version 7.0).

Equation 2-3. Equation for calculating Pearson correlation coefficient, where $\bar{x}$ and $\bar{y}$ are sample means of each variable.

$$\text{Pearson correlation coefficient} = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2\,\Sigma(y-\bar{y})^2}}$$

Equation 2-4. Equation for calculating one sample t-test (t), where $\bar{x}$ is sample mean, *μ* is assumed population mean, *s* is sample standard deviation and *n* is sample size.

$$t = \frac{\bar{x} - \mu}{\frac{s}{\sqrt{n}}}$$

Grubbs' test (G, $p < 0.05$), shown in Equation 2-5, was used for checking data for outliers. If the G value calculated for a suspected outlier was greater than the value from the Grubbs' table at the 95% confidence level, it was identified as an outlier and was rejected from the dataset.

Equation 2-5. Equation for performing Grubbs' test, where $\bar{x}$ is sample mean, *x* is a suspected outlier and *s* is sample standard deviation.

$$G = \frac{|\bar{x} - x|}{s}$$

Bland–Altman analysis was performed in GraphPad Prism software (version 7.0) to assess the agreement between analytical methods.

2.3 Pharmacokinetic calculations

Pharmacokinetic data for mephedrone and its metabolites was determined both manually and by using a non-compartmental pharmacokinetics data analysis program (PK Solutions, version 2.0, Summit Research Services, Montrose, Colorado). Obtained data from the software was compared with the manually calculated data to ensure they were in agreement.

Peak concentration (C_{max}) and the time after dosing when it occurred (T_{max}) were observed directly from the data for mephedrone and its metabolites. The apparent elimination half-life ($t_{1/2}$) was calculated using a log-linear regression of the elimination phase by plotting drug concentration versus time using data points which produced R^2 of at least 0.97. The elimination rate constant (k_{el}) was calculated using Equation 2-6.

Equation 2-6. Equation for calculating the elimination rate constant (k_{el})

$$k_{el} = \frac{\ln(2)}{t_{1/2}}$$

The area under the curve (AUC) from 0 to Day 3 or Day 4 was calculated using the trapezoidal method. Clearance (CL) was calculated using Equation 2-7 and was then adjusted for the individual's weight. Renal clearance (CL_r) was calculated as shown in Equation 2-8.

Equation 2-7. Equation for calculating apparent oral clearance (CL)

$$CL = \frac{\text{dose}}{AUC}$$

Equation 2-8. Equation for calculating renal clearance (CL_r)

$$CL_r = \frac{total\ mephedrone\ eliminated\ in\ urine}{AUC}$$

The apparent volume of distribution (V) was calculated using Equation 2-9 and was then adjusted for the individual's weight.

Equation 2-9. Equation for calculating volume of distribution (V)

$$V = \frac{CL}{k_{el}}$$

2.4 Method development

2.4.1 Column selection

Separation of mephedrone in biological samples is usually performed on a reverse phase liquid chromatography (RPLC) which provides favourable interactions between non-polar analytes and hydrophobic stationary phase. However, for the purpose of this project a suitable chromatographic method had to be developed allowing separation not only of mephedrone but also its metabolites which exhibit a range of polarities. Hydroxytolyl-mephedrone and 4-carboxy-mephedrone, two more polar metabolites, are poorly retained on a standard C18 stationary phase. These metabolites tend to co-elute with matrix interferences shortly after the start of the gradient which results in loss of sensitivity and inaccurate quantification. One way to overcome this problem and to improve the retention of polar analytes is to use highly aqueous mobile phase at the beginning of the gradient, but this approach leads to poor desolvation and ion suppression. Moreover, traditional reverse phase columns are not compatible with highly aqueous mobile phases (> 95% water) due to of a phenomenon known as 'phase collapse', where hydrophobic hydrocarbons on the stationary phase tend to 'collapse' onto themselves to avoid contact with a highly polar mobile phase, leading to poor column efficiency. Newer types of analytical columns incorporate polar end-capping groups, rendering stationary phases water wettable. It was therefore decided to test both: Waters ACQUITY HSS T3 column (2.1 mm x 150 mm, 1.8 µm) which can withstand

100% aqueous mobile phases, and a fully end-capped UCT Selectra® pentafluorophenylpropyl (PFPP) column (2.1 mm x 150 mm, 1.8 µm) which should provide more interactions with the polar metabolites.

Chromatographic separation on both columns was performed on a Waters Acquity UPLC system equipped with a 2777 open architecture autosampler coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Mobile phase A was 0.3% formic acid in water and mobile phase B was 0.3% formic acid in acetonitrile. Strong needle wash was methanol and weak needle wash was acetonitrile:water (10:90 v/v). PFPP column was held at 60°C with the flow rate of 0.5 mL/min. The start of the gradient was at 85% mobile phase A. Mobile phase B was then increased to 55% over 11 min and was held for 2 min. Over the next 0.5 min the gradient returned to the starting condition and the column was re-equilibrated at 85% mobile phase A for the remaining 1.5 min. The total run time was 15 min. The HSS T3 column was held at 40°C with a flow rate of 0.4 mL/min and a total run time of 20 min. The initial gradient was 100% mobile phase A for 0.5 min and was then decreased to 20% mobile phase A over 17.5 min. Over the next 0.5 min the gradient returned to 100% mobile phase A for 2 min and was held there for 1.5 min. Twenty microliters of extracted whole blood (see section 2.4.4 for more information about the extraction) samples (n=6) was injected on both columns using a 100 µL syringe with a 10 µL injection loop (full loop injection with loop overfill). The data was acquired and processed using MassLynx software (version 4.1).

As shown in Table 2-3, all analytes were retained on both columns, however, PFPP column provided significantly improved retention. In addition, unexpected retention time shifts of approximately 0.25 min were observed for hydroxytolyl-mephedrone and 4-carboxy-mephedrone in 2 out of 6 injections of the same sample on the HSS T3 column. As a result, PFPP column was chosen for analysis.

Table 2-3. Comparison of the retention times on the HSS T3 and PFPP columns

Analyte	Retention time on HSS T3	Retention time on PFPP
Mephedrone	3.71 min	5.85 min
Dihydro-mephedrone	3.42 min	5.38 min
Nor-mephedrone	3.25 min	5.00 min
Hydroxytolyl-mephedrone	1.25 min	1.98 min
4-carboxy-mephedrone	1.26 min	2.06 min
Dihydro-nor-mephedrone	2.97 min	4.45 min

2.4.2 Mass spectrometry

Mass spectrometry (MS) analysis was performed using a Waters Xevo TQ-S triple quadrupole mass spectrometer equipped with a Z-spray electrospray ionisation source operated in positive ion mode. The following analytes were targeted: mephedrone hydrochloride (MEPH), dihydro-mephedrone hydrochloride (DHM), mephedrone-d_3 hydrochloride (MEPH-d_3), dihydro-mephedrone-d_3 hydrochloride (DHM-d_3), 4-(2-aminoethyl) benzoic acid hydrochloride (AEBA), nor-mephedrone hydrochloride (NOR), hydroxytolyl-mephedrone hydrochloride (HYDROXY), 4-carboxy-mephedrone hydrochloride (4-CARBOXY) and dihydro-nor-mephedrone (DHNM).

All analytes were individually diluted in 0.3% formic acid (FA) in acetonitrile:water (50:50 v/v) to yield 10 ng/mL solutions. These solutions were infused and the method was optimised to give the following conditions: the source temperature was set to 150°C, desolvation gas flow rate was 1000 L/h at a temperature of 500°C, capillary voltage was set to 2.22 kV, cone voltage was 45 V, source offset was 84 V, cone gas flow rate was set to 150 L/h, the nebulizer gas flow was 7.00 bar and the collision gas flow rate was 0.25 mL/min.

In addition, product ion scans were performed and collision energies (CE) optimised to yield the most abundant product ions (1 quantifier and 2 qualifier ions). These were then used to create two selected reaction monitoring (SRM) methods: one for the intact ions and the other one for the ions that gave water losses. Both SRM methods were compared in terms of generated peak intensities. Even though the SRM method based on the *m/z* values that included the in-source water loss generated less fragmentation (because of the lower *m/z*), it produced higher signal intensity and was therefore chosen as a preferred SRM method.

SRM used to monitor analytes and internal standards are listed in Table 2-4. In addition to MEPH-d_3 and DHM-d_3, AEBA was used as an internal standard for 4-CARBOXY (whole blood only) to help minimise matrix effects.

Table 2-4. SRM transitions and CE for each ion

** denotes a quantifying transition*

*** denotes dehydrated precursor ions*

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)
MEPH	160.4 **	145.1*	15
		144.1	33
		91.1	28
MEPH-d_3	163.4 **	148.4	19
DHM	162.4 **	147.3*	19
		131.4	17
		91.3	26
DHM-d_3	165.4 **	150.3	18
NOR	146.0 **	131.1	25
		130.1*	25
		119.0	15
HYDROXY	194.1	158.1	17
		146.0*	17
		131.1	23
4-CARBOXY	208.0	146.0*	13
		144.1	28
		130.1	31
DHNM	148.1 **	131.1*	13
		116.2	23
		91.1	25
AEBA	166.1	149.1	10

2.4.3 Synthesis of dihydro-nor-mephedrone

DHNM, one of MEPH metabolites, was not commercially available at the time of this research. NOR was therefore used as a starting material for the synthesis of DHNM

(Figure 2-1). Ten milligrams of NOR (61.3 µmol) was reduced to DHNM following a method described elsewhere [387]. Synthesised product was stored at -40°C and a small amount was analysed by high resolution MS (HRMS) on ThermoFisher Scientific Q-Exactive operated in positive mode to determine its accurate mass. Proton nuclear magnetic resonance (^{1}H NMR) was also performed on a Bruker Avance DRX 400 MHz instrument.

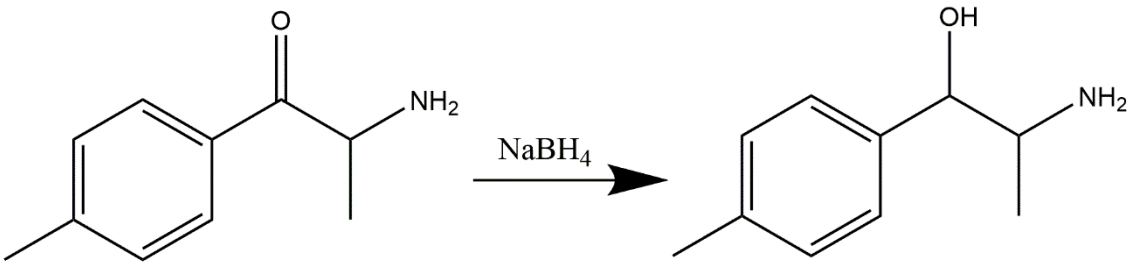


Figure 2-1. Synthesis of dihydro-nor-mephedrone from nor-mephedrone

DHNM was successfully synthesised (yield: 51%). Formula $C_{10}H_{16}NO$; HRMS [M+H$^+$] calculated m/z 166.1226, observed 166.1227. Observed MS/MS fragments with collision energy 20 eV were consistent with those reported in the literature [74]. ^{1}H NMR ($CDCl_3$) δ 7.22 (d, J=8.0 Hz, 2H, Ar-H), 7.16 (d, J=8.0 Hz, 2H, Ar-H), 4.50 (d, J=4.0 Hz, 1H, CH(OH)), 3.19 (s, 1H, $CH(CH_3)$), 2.35 (s, 3H, Ar-CH_3) and 0.98 (d, J=8.0 Hz, 3H, $CH(CH_3)$).

2.4.4 Solid phase extraction: whole blood, plasma, urine

Because at the beginning of the project, MEPH and DHM were the only affordable and easily accessible reference standards, initial method development was limited to these two analytes. Other reference standards required custom synthesis and were received at different points in time. As a result, some method development experiments were initially not performed with all analytes.

2.4.4.1 General approach

Because MEPH and its metabolites exhibit a wide range of physico-chemical properties (Table 2-5), XtrackT® DAU High Flow (150 mg, 3 mL) solid phase extraction (SPE) cartridges were chosen for sample preparation. XtrackT® cartridges contain C8 hydrophobic chains and benzoylsulfonate anions facilitating mixed mode reverse-phase/cation exchange, which allows simultaneous retention of non-polar and polar analytes.

Table 2-5. pKa and logP values generated by the Marvin software (version 17.16.0) for mephedrone and its metabolites

Analyte	pKa	logP
MEPH	8.03	2.12
DHM	9.55	1.83
NOR	7.57	1.69
HYDROXY	8.01	0.84
4-CARBOXY	3.64 (acid), 8.03 (amine)	-1.20
DHNM	9.39	1.40

The use of mixed mode SPE for an extraction of a wide range of drugs is a well-established approach in analytical toxicology [388]. The stationary phase is firstly conditioned with methanol and equilibrated with phosphate buffer$_{(aq)}$ pH 6.0. After sample loading step, water is added to remove polar interferences which is followed by the addition of diluted acetic acid$_{(aq)}$, pH 3.3 to ionise basic functional groups. At this point basic analytes are 'locked', interacting with hydrophobic C8 chains and benzoylsulfonate anions, as shown in Figure 2-2. Methanol wash can also be employed at this stage to remove neutral matrix components. Samples are then dried to remove residual aqueous solution and analytes are eluted. Acidic and neutral analytes are usually eluted with an organic solvent which disrupts hydrophobic interactions whereas

basic analytes are eluted with an organic solvent containing ammonium ions which disrupt ionic interactions. If required, neutral and basic analytes can be eluted at the same time.

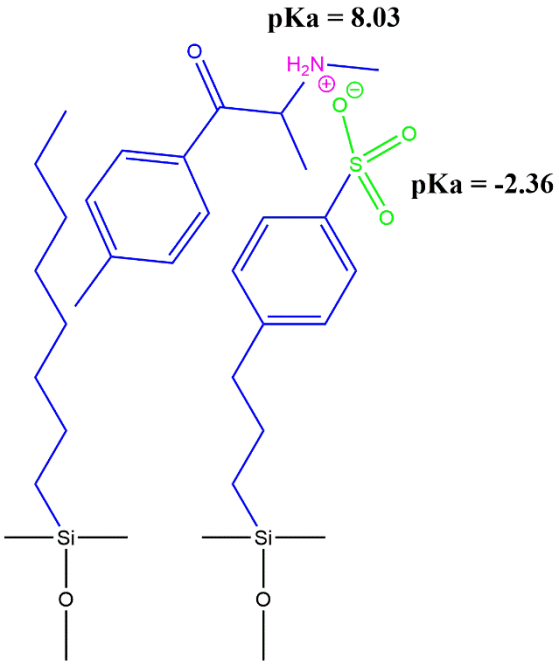


Figure 2-2. Ionic and hydrophobic interactions between protonated mephedrone and a mixed mode sorbent in the 'acid-lock' step

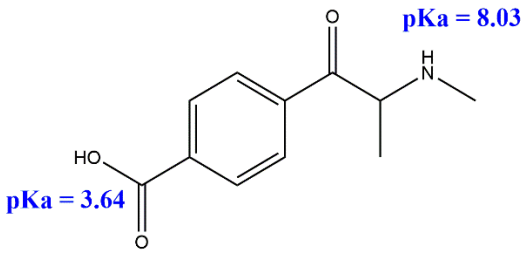


Figure 2-3. Chemical structure of 4-CARBOXY and its pKa values

2.4.4.2 Water wash step

SPE optimisation began with a variation of the general mixed-mode SPE extraction described in 2.4.4.1. Whole blood samples prepared at 500 pg/mL were vortex mixed and 1 mL of 0.1 M phosphate buffer, pH 6.0 was added. After conditioning XtrackT® cartridges with 2 mL of methanol and 2 mL of 0.1 M phosphate buffer (pH 6.0), samples were loaded and washed with 2 mL of 0.1 M acetic $acid_{(aq)}$ followed by 2 mL of water and 2 mL of methanol. Samples were finally eluted with 4 x 1 mL of dichloromethane (DCM) : propanol (IPA) : ammonium hydroxide (78:20:2 v/v/v).

It was noted that compared to other analytes, 4-CABROXY resulted in poor recovery (14.5 ± 9.96%). 4-CARBOXY is a zwitterionic compound (see Figure 2-3) which contains an acidic carboxylic acid group and a basic secondary amine group. As shown in Table 2-5, the secondary amine has a pKa of 8.03 and the carboxylic acid has a pKa of 3.64. At the sample load stage (pH 6.0) both functional groups are ionised and so the compound exists as a zwitterion with a net neutral charge, being retained by the π-π interactions between the benzene ring on 4-CARBOXY and hydrophobic carbon chains of the sorbent. It was hypothesised that the first SPE wash, which was the addition of 2 mL of water, washed the analyte off the sorbent. The removal of the water wash has not significantly affected the recoveries of other analytes, but the recovery of 4-CARBOXY more than doubled albeit still remained relatively low compared to other analytes (Table 2-6). This may be because only about 80% of the carboxylic acid group is protonated upon the addition of diluted acetic acid (pH 2.9) during the second wash step in SPE. As a result, the remaining 20% of the molecules, which exist as a neutral zwitterion with no net charge, are removed following a methanol wash. The methanol wash disrupts the hydrophobic interaction of the 'net neutral' 4-CARBOXY with the hydrophobic carbon chains, causing a considerable proportion to be lost. The other analytes are all basic and as cations ionically interact with the benzoylsulfonate anion within the mixed mode stationary phase during the methanol wash, which results in higher recovery. As a result of this experiment, water wash step was excluded from the final SPE method.

Table 2-6. Analyte recovery ± %CV under different SPE conditions

SPE condition	MEPH	DHM	4-CARBOXY
With water wash	88.9 ± 8.27%	89.5 ± 5.76%	14.5 ± 9.96%
Without water wash	84.7 ± 0.171%	83.8 ± 1.90%	34.0 ± 2.00%

2.4.4.3 Protein precipitation before solid phase extraction

As described in 1.3.1, sample treatment is performed to remove proteins, salts or other matrix components which could not only act as chromatographic interferences or

precipitate out in a LC system causing blockages but could also form drug-protein complexes making total drug quantification inaccurate. Besides sample centrifugation and filtration, protein precipitation with organic solvents is the most widely used technique. An organic solvent, such as acetonitrile (ACN), displaces water molecules from the hydrophobic regions on the protein surface, thus decreasing its solvation layer. Increased electrostatic and dipole forces cause proteins to aggregate and precipitate out of the solution [389]. For the protein precipitation experiment, 400 µL of ACN was added to 100 µL whole blood samples prepared at 500 pg/mL. Samples were then mixed for 10 min at 1400 rpm and centrifuged at 13,200 g for 5 min. The supernatant was transferred to a clean tube and the solvent was evaporated at 45°C in an Eppendorf Concentrator Plus®. Following the addition of 1 mL of 0.1 M phosphate buffer (pH 6.0) samples were taken through the SPE as described in 2.4.4.2.

Table 2-7 compares the effect of sample treatment with the ACN (n=3) and without (n=3). Higher recoveries and better precision were observed for all analytes following the extraction procedure which did not involve protein precipitation (PPT). The extent of mephedrone (and its metabolites) binding to plasma proteins has not been determined in humans but mephedrone-protein binding has been shown to be 21.6 ± 3.67% in Sprague-Dawley rats [92]. If similar extent of protein binding occurs in humans, protein precipitation should in theory disrupt analyte-protein binding, leading to better recoveries. This was not the case here, which might suggest that the analytes are lost in the precipitate or other factors are at play.

Table 2-7. Analyte recovery ± %CV following sample treatment with a protein precipitation solvent (n=3) and without (n=3)

	MEPH	DHM	NOR	4-CARBOXY	DHNM
No PPT	91.8 ± 2.99%	78.8 ± 2.09%	87.8 ± 5.25%	32.9 ± 5.55%	86.6 ± 3.08%
PPT with ACN	75.7 ± 28.5%	57.7 ± 7.55%	60.4 ± 5.35%	21.8 ± 13.8%	65.8 ± 7.85%

2.4.5 Solid phase extraction: oral fluid

Method validation and oral fluid sample analysis was carried out at Abbott (previously Alere Toxicology). Their in-house validated method for the detection of stimulant drugs (including mephedrone) was followed during sample analysis which meant that method development was not necessary. Extraction and instrumental details are detailed in Section 6.2.3 in Chapter 6.

2.4.6 Chiral separation in whole blood

Enantiomers usually differ in their biochemical activity, which may result in one enantiomer being biologically active while the other one is inactive or may even produce different/adverse effects. Enantiomers are commonly separated by chiral chromatography with the use of chiral stationary phases or mobile phase additives [390]. However, chiral method development is time consuming whereas chiral columns are expensive and lose their resolving power over time [391]. A different approach relies on reacting enantiomers with an optically pure reagent to form diastereoisomers. Unlike enantiomers, diastereoisomers have different chemical and physical properties which means that they can be separated on achiral stationary phases.

In addition to the techniques described above, circular dichroism (CD) spectroscopy is often used to distinguish enantiomers based on their differential absorption of left- and right-handed circularly polarised light [391]. An optically pure compound will rotate polarised light which results in a signal in the CD spectrum. The signal shows the extent to which the polarised light is rotated after passing through the sample [390], with a pair of enantiomers resulting in a mirrored CD spectrum.

2.4.6.1 Supercritical fluid chromatography

Supercritical fluid chromatography (SFC) uses a supercritical fluid, which can be any substance as long as it is heated above its critical temperature and compressed above

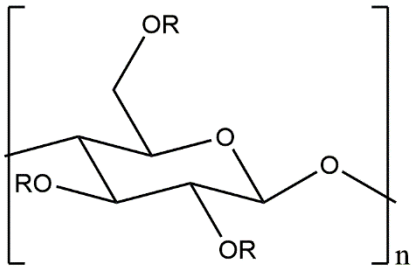
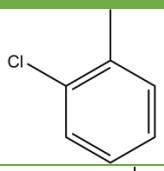
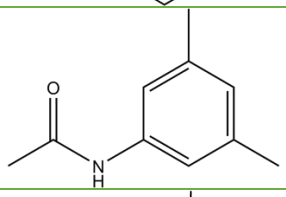
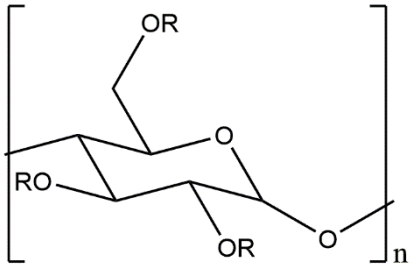
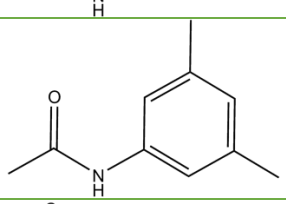
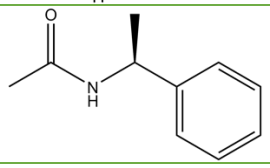
its critical pressure. Due to low critical pressure (73 atm) and critical temperature (31°C), low cost and low toxicity, carbon dioxide (CO_2) is the most commonly used for this purpose [392]. Because CO_2 on its own is not polar enough to elute certain analytes, organic modifiers (such as methanol and acetonitrile) are often added to increase the polarity of the mobile phase, and thus to improve solubility of polar analytes. In addition, acidic or basic modifiers such as, dimethylamine, formic acid or trifluoroacetic acid can also be added to improve retention time and resolution. Even though the use of the additives and modifiers may extend the application of SFC, it is important to carefully consider the compatibility of additives with the detector and their potential impact on ionisation efficiency [393]. SFC is predominantly coupled to MS but other detection methods include ultraviolet-visible spectroscopy, ultraviolet-visible spectrophotometry and flame ionization detector.

Higher flow rates made possible by lower system pressure exerted by the supercritical fluid and optional additives deliver a fast and efficient separation without losses in resolution or efficiency [394]. It has been shown that SFC provides at least two times faster separation than normal phase LC [395,396], which is one of the reasons why it has been used in pharmaceutical industry and drug discovery for the separation of racemic mixtures on chiral columns. Moreover, fraction collection with SFC does not require long drying times because CO_2 evaporates quickly leaving behind analyte(s) and a small volume of organic additive. On the other hand, limited choice of mobile phases, reliance on bulky CO_2 cylinders and unwanted reactions with the mobile phase may become problematic.

The aim of this part of the project was to develop a method that would separate enantiomers of mephedrone and some of its metabolites. SFC coupled to diode array detection (DAD) was initially employed for this task. A selection of chiral columns (see Table 2-8) was screened with mobile phases containing trifluoroacetic acid (TFA) and isopropylamine as additives as well as propanol (IPA) or methanol (MeOH) as modifiers. In addition, run times, flow rates and mobile phase composition were varied and tested. As (S) and (R) enantiomers of mephedrone were not commercially available, pure

enantiomers of mephedrone analogues (cathinone and methcathinone) were run alongside mephedrone to indicate likely elution order of mephedrone enantiomers.

Table 2-8. A list of chiral columns screened on SFC-DAD

Stationary Phase	R=	Column details
		PHENOMENEX **LUX-CELLULOSE-2** 150 mm x 2 mm i.d., 3 µm
		DAICEL CHIRALPAK **OD-3** 150 mm x 3 mm i.d., 3 µm
		DAICEL CHIRALPAK **AD-3** 150 mm x 3 mm i.d., 3 µm
		DAICEL CHIRALPAK **AS-3** 150 mm x 2.1 mm i.d., 3 µm

After an extensive evaluation, only CHIRALPAK OD-3 column with 0.1% TFA and 0.1% isopropylamine in IPA as organic modifiers/additives separated mephedrone enantiomers in a 20 min run time (see Figure 2-4). During the isocratic run CO_2 was kept at 97.5%, column temperature was 40°C and the flow rate was 1 mL/min. Mobile phase prepared with only TFA or isopropylamine did not result in a separation which is in agreement with previous research where the combination of both additives was reported to give broader enantioselectivity of acidic, basic and neutral compounds [397]. Basic amines, such as isopropylamine, mask the effects of the charged silanol groups which often result in a poor peak shape and low resolution when left available for non-specific retention of basic compounds [394]. In addition, basic amines also provide interaction with neutral chiral selectors by neutralising charged groups on basic analytes [393]. Acidic additives, such as TFA, protonate amino groups leading to increased

interactions with the carbamate groups on the stationary phase, thus improving enantioselectivity [397].

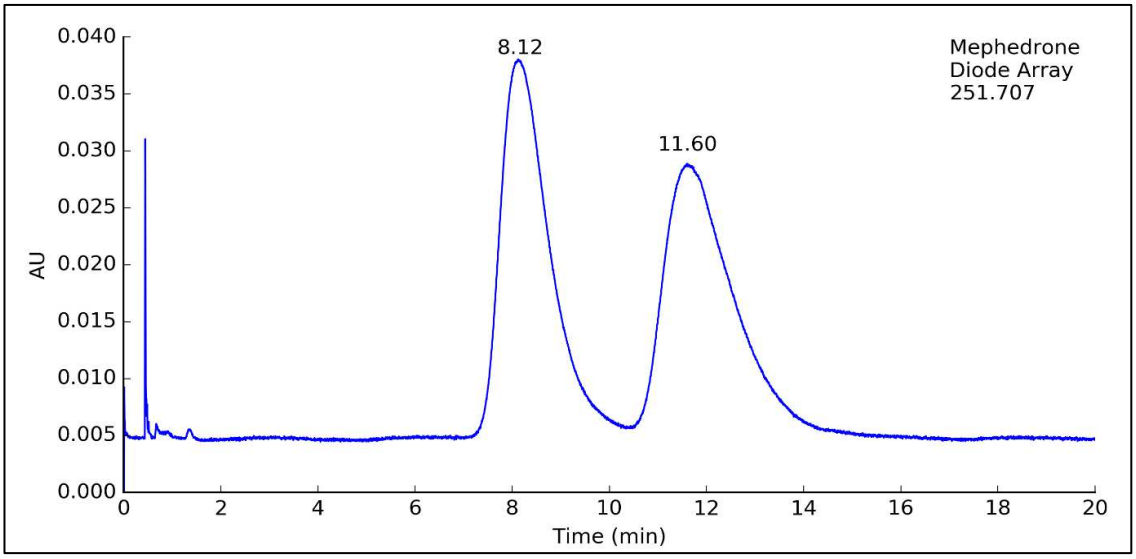


Figure 2-4. Separated mephedrone enantiomers on the CHIRALPAK OD-3 column on SFC-DAD

2.4.6.2 GC-MS method on an achiral column

Even though a desired separation was achieved, the reliance of separation on isopropylamine and TFA made the method unsuitable for coupling with a MS detector. TFA is a known ion-paring agent lingering in the LC-MS system and contributing to high background noise and signal suppression [398]. In addition, isopropylamine has been previously reported to have negative impact on the MS detection [399]. As a result, a new strategy of mephedrone derivatisaion with two enantiomerically pure chiral derivatisation reagents: (S)-(−)-N-(Trifluoroacetyl) pyrrolidine-2-carbonyl chloride (L-TPC) and (R)-(−)-α-Methoxy-α-(trifluoromethyl) phenylacetyl chloride (MTPA) to produce diastereoisomers of mephedrone (Figure 2-5) was investigated. Diastereoisomers have different chemical and physical properties and can be separated on achiral stationary phases.

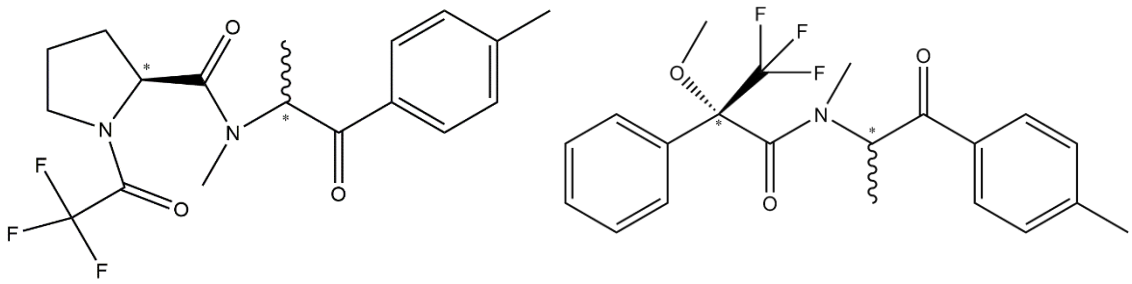


Figure 2-5. L-TPC-mephedrone (left) and MTPA-mephedrone (right)

Derivatisation was performed following a method described in the literature [400]. One hundred microliters of 50 µg/mL solution of mephedrone prepared in water was aliquoted into a vial together with 125 µL of saturated aqueous solution of potassium carbonate, 1.5 mL of ethyl acetate and 12.5 µL of L-TPC or MTPA. Samples were stirred for 10 min at room temperature. The upper layer was transferred into a new vial and dried with anhydrous sodium sulfate. A small aliquot was then injected onto GC-MS following a method published before [401].

As shown in Figure 2-6, diastereoisomers formed by both derivatisation reagents (L-TPC and MTPA) were separated on GC-MS, but different peak height ratios were observed. Diastereoisomers of L-TPC-mephedrone produced a ratio of about 2:1 (different to the separation achieved on SFC-DAD) whereas diastereoisomers of MTPA-mephedrone resulted in a more equal ratio of nearly 1:1. L-TPC is a commonly used derivatisation reagents for analytes containing a primary or secondary amine group. However, it has been reported to undergo racemisation due to keto-enol tautomerism of the α-proton on the chiral carbon and the carbonyl group [400]. In contrast, MTPA does not have an α-proton on the chiral carbon and should therefore be more stable and produce more reliable peak height/area ratios [402], which in this case were similar to the enantioseparation obtained on SFC-DAD.

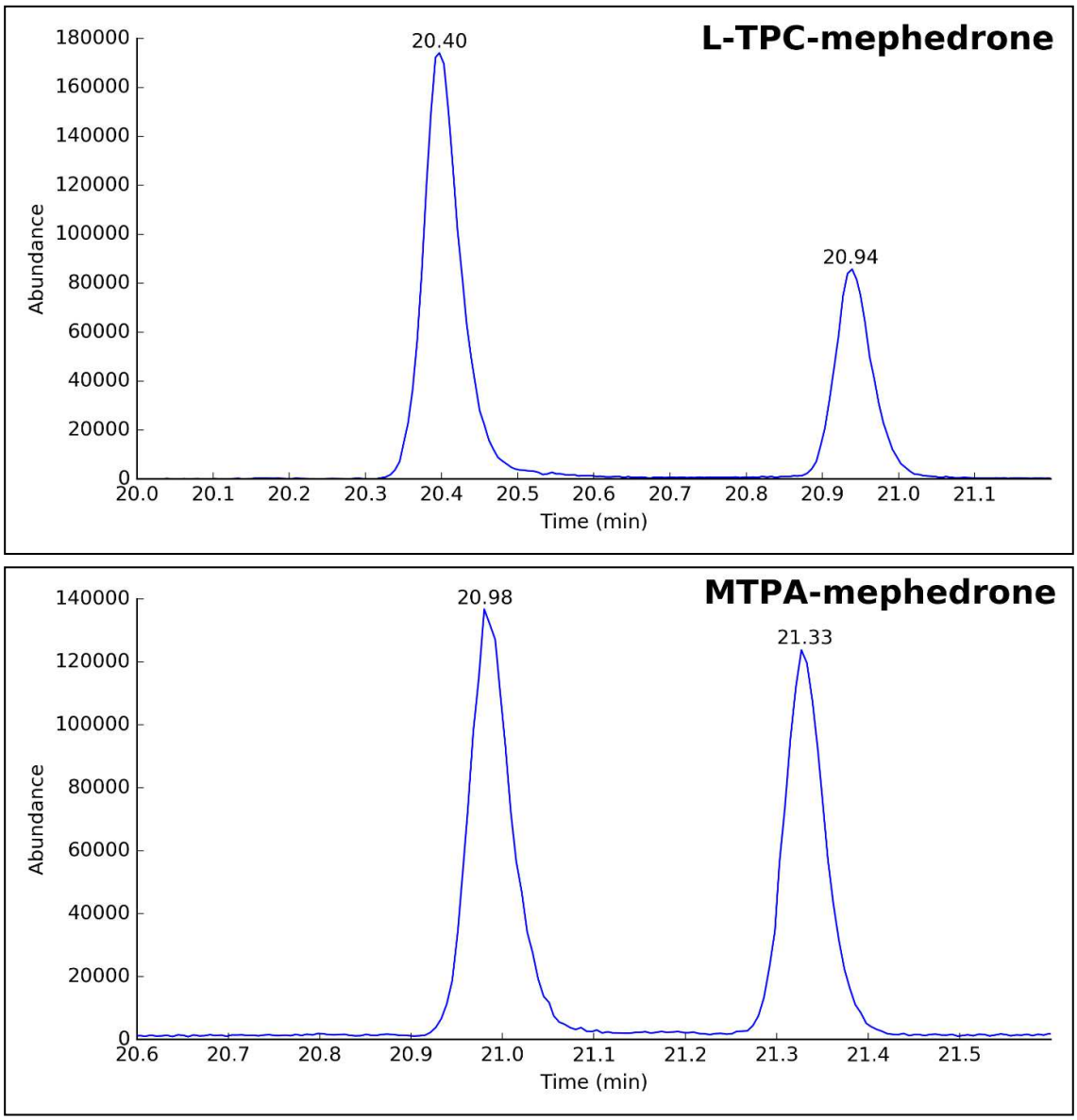


Figure 2-6. Separation of diastereoisomers of mephedrone on GC-MS after derivatisation with MTPA (bottom) and L-TPC (top)

2.4.6.3 LC-MS method on an achiral column

Because several methods aimed at quantification or detection of the enantiomers of synthetic cathinones by GC-MS had been published, it was recognised that a novel aspect with regards to this project would have to focus on the quantification of mephedrone enantiomers on a chiral column on a LC-MS system. Derivatisation products described in 2.4.6.2 were infused on a Waters Xevo TQ-S triple quadrupole and

SRM transitions were created. Following LC optimisation, L-TPC-mephedrone was partially separated on a UCT Selectra DA® column, but a 3:1 peak height ratio was observed. No separation was achieved for MTPA-mephedrone.

A different chiral derivatisation reagent, N-(2,4-dinitro-5-fluorophenyl) L-valinamide (DNPV), was found to have been previously reported to aid stereoselective detection of MDMA enantiomers as well as its Phase I and Phase II metabolites [403]. As a final effort 20 µg/mL of mephedrone alongside 20 µg/mL of MDMA (which served as a positive control) were derivatised with 100 µL of DNPV (0.3% in acetone w/v) in the presence of 100 µL of 0.1 M carbonate buffer (pH 9.0). After 30 min of mixing at 50°C at 1200 rpm, the reaction was stopped by adding 20 µL of 1 M $HCl_{(aq)}$. Samples were diluted with the mobile phase and were infused. DNPV-mephedrone was not detected, demonstrating unsuccessful derivatisation. It was speculated that DNPV was unable to react with the secondary amine on mephedrone due to steric hinderance caused by the ketone group, which is not present in MDMA (see Figure 2-7). DNPV-MDMA produced a strong signal at m/z 474 which was in agreement with the literature [403].

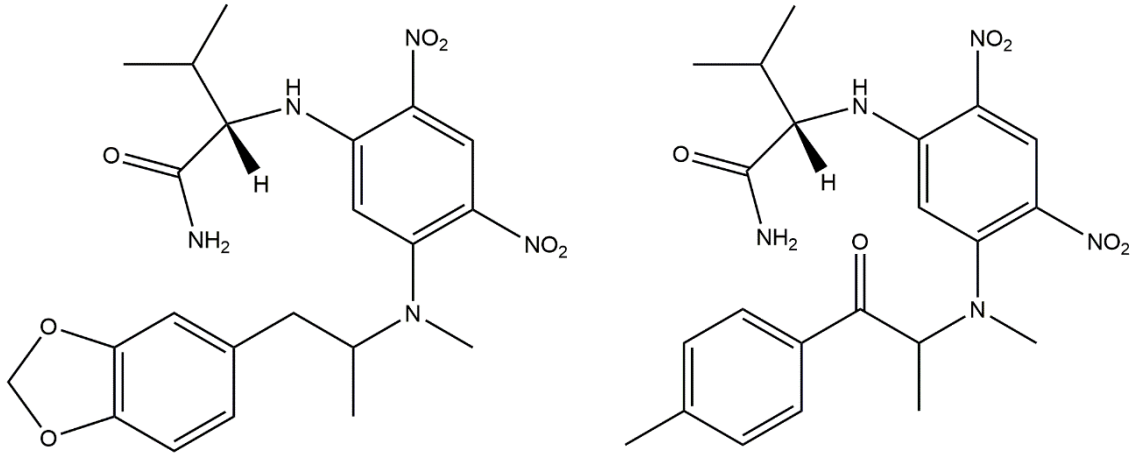


Figure 2-7. DNPV-MDMA (left) and a proposed structure of DNPV-mephedrone (right)

2.4.6.4 HPLC-DAD method on a chiral column

At this stage the literature was reviewed, and a decision was made to screen chiral columns on a HPLC-DAD system. An instruction manual for CHIRALPAK AD columns suggested using MeOH:ethanol (50:50 v/v) as a mobile phase in an isocratic elution mode and a basic modifier, for example diethylamine (DEA). These conditions gave a partial separation of mephedrone and NOR enantiomers which were eventually fully resolved by changing the ratio of the alcohols to MeOH:ethanol (80:20 v/v, see Figure 2-8). MEPH-d_3 was also successfully separated under these conditions (data not shown). The method was then transferred onto LC-MS and used for chiral whole blood analysis.

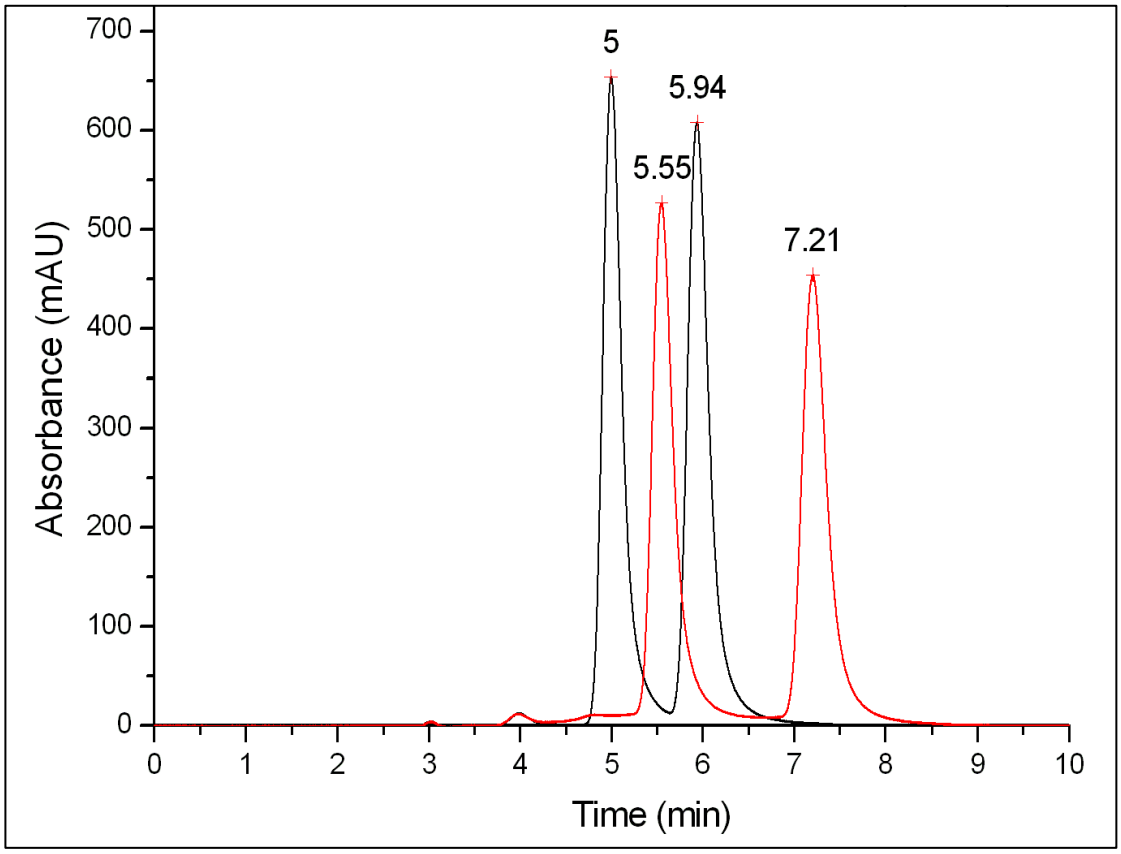


Figure 2-8. Separation of mephedrone (black) and NOR (red) enantiomers on the CHIRALPAK AD-3 column with 0.1% DEA in MeOH:ethanol (80:20 v/v) as mobile phase on HPLC-DAD

2.4.6.5 Determination of the elution order

With the enantiomeric separation achieved on the LC-MS system, the remaining task was to determine the elution order of mephedrone enantiomers (the elution order of NOR enantiomers was not investigated because the method was not sensitive enough to detect NOR in whole blood samples from the administration study). Mephedrone enantiomers were separated on a semi-preparative column (Lux® 5 µm Amylose-1, 250 x 10 mm), which is equivalent to CHIRALPAK AD-3, on Agilent HPLC 1050 system equipped with a manual injector coupled to DAD. Two injections of the mephedrone solution prepared at 5 mg/mL were performed and are referred to here as F1 and F2. Manually collected fractions were diluted 1 in 10 in the mobile phase and injected on the CHIRALPAK AD-3 column. This was done to select fractions containing only one pure enantiomer. Only the second eluting peak/enantiomer was isolated in pure form and brought forward for further characterisation. Pure fractions were dried overnight, dissolved in MeOH and combined.

CD spectra were acquired on the Applied Photophysics Chirascan Plus spectrometer (Leatherhead, UK). One millimetre (Hellma UK) Quartz Suprasil rectangular cells were used in the region of 450-180 nm. The instrument was flushed continuously with pure evaporated nitrogen throughout the experiment. The following parameters were employed: 2 nm spectral bandwidth, 1 nm stepsize and 1.0 s accumulation time per point. The CD spectra were solvent baseline corrected and measured at +23°C. The CD spectra were smoothed using the Savitzky-Golay method for better presentation. Data processing was done using APL Prodata Viewer (version 4.2.15) and spectra were modified in Origin (version 6.0).

Alongside collected fractions (F1 at 1 mg/mL and F2 at 0.5 mg/mL), 1 mg/mL solutions of (R)-methcathinone and (S)-methcathinone were also analysed. Methcathinone differs from mephedrone by the absence of a methyl group on the benzene ring. Because the rest of the structure, including the chemical environment surrounding the chiral centre,

is identical, CD spectra of F1 and F2 should reveal the correct mephedrone enantiomer based on the reference CD spectra obtained by analysing methcathinone enantiomers.

Figure 2-9 shows the CD spectrum of (S)-methcathinone and (R)-methcathinone, clearly demonstrating their enantiomeric nature. Compared to the spectrum in Figure 2-9, it can be observed that F1 and F2 (containing the second eluting peak in the chiral LC method) in Figure 2-10 correspond to the (S)-enantiomer with the CD spectrum transitioning from negative to positive CD values. This means that the first eluting peak in Figure 2-8 can be attributed to (R)-mephedrone and the second eluting peak is (S)-mephedrone. Of note, racemic mephedrone did not yield any significant peak due to the balancing effects of the equally contributing enantiomers.

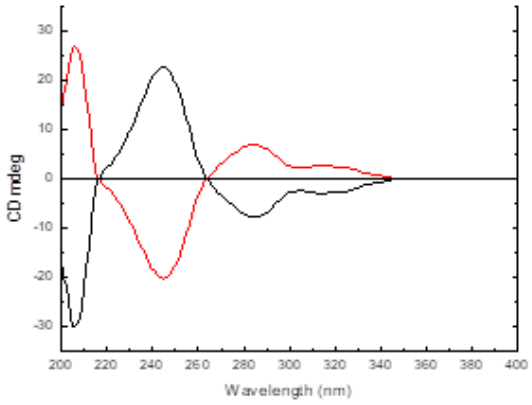


Figure 2-9. CD spectrum of (S)-methcathinone (black) and (R)-methcathinone (red)

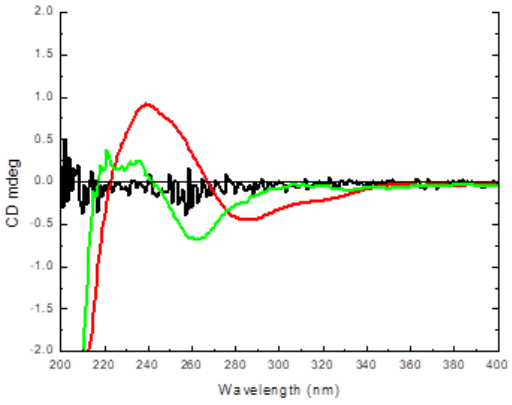


Figure 2-10. CD spectrum of racemic mephedrone (black), F1 (red) and F2 (green)

2.4.7 Dried blood spots

2.4.7.1 Recovery with different extraction solvents

Ten microliter Mitra® devices were dipped in either drug-free whole blood or in whole blood spiked with all analytes at 10 ng/mL. Mitra® devices were left to dry at room temperature for 2 h. Tips were then transferred into clean Eppendorf tubes and a range

of extraction solvents was tested: MeOH, ACN, ACN:H_2O (50:50 v/v), 1% ammonium hydroxide in MeOH:ethyl acetate (50:50 v/v, MeOH:EtOAc), 1% NH_4OH in ACN:H_2O (50:50 v/v), ACN:H_2O (70:30 v/v), 0.3% FA in MeOH, MeOH:2% zinc sulphate ($ZnSO_4$), 8% $ZnSO_{4(aq)}$ and saturated $ZnSO_{4(aq)}$. Samples were sonicated for 15 min at 35kHz and vortex mixed for 5 min at 1300 rpm. Solvent was then transferred to a new Eppendorf tube, leaving the tip behind, and was evaporated under vacuum at 45°C in an Eppendorf Concentrator Plus®. Spiked whole blood samples were reconstituted with 100 µL of 0.1% FA in ACN:H_2O (10:90 v/v) whereas drug-free whole blood samples were reconstituted with 100 µL of a solution containing all analytes prepared in 0.1% FA in ACN:H_2O (10:90 v/v).

Based on the results presented in Figure 2-11 and Figure 2-12, 0.3% FA in MeOH gave the best recovery for all analytes, except MEPH which had better recovery in pure MeOH. In contrast, ACN gave the lowest recoveries for all analytes, with 4-CARBOXY not being detected in these samples at all. Zinc sulphate-based solvents extracted no more than 50% of the analytes.

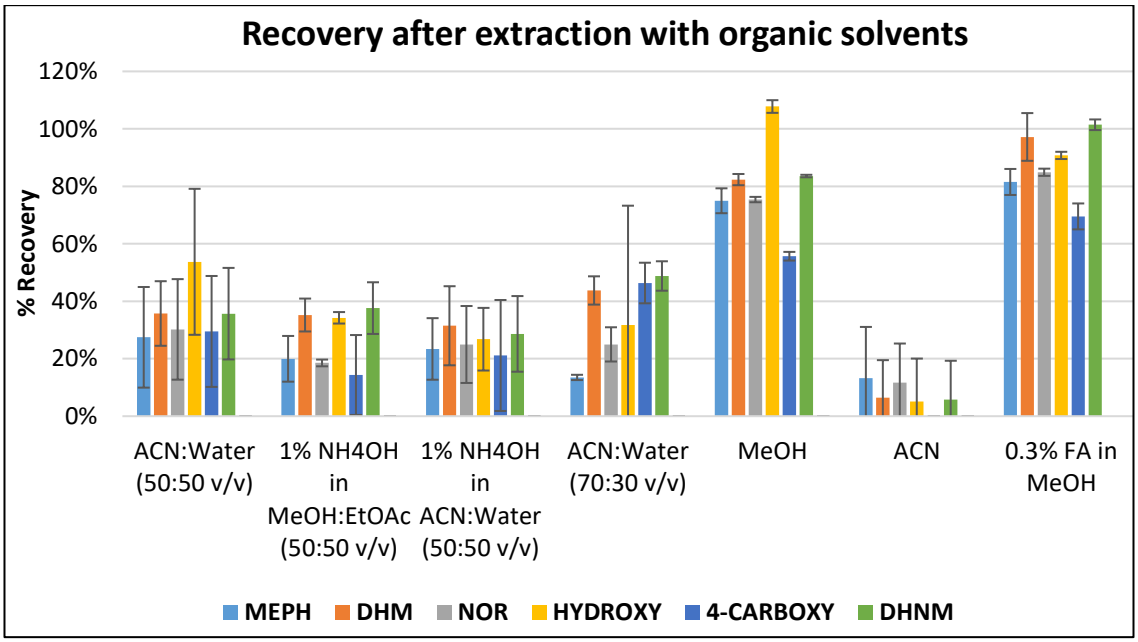


Figure 2-11. Recovery ± %CV for each analyte after an extraction with organic solvents (n=3)

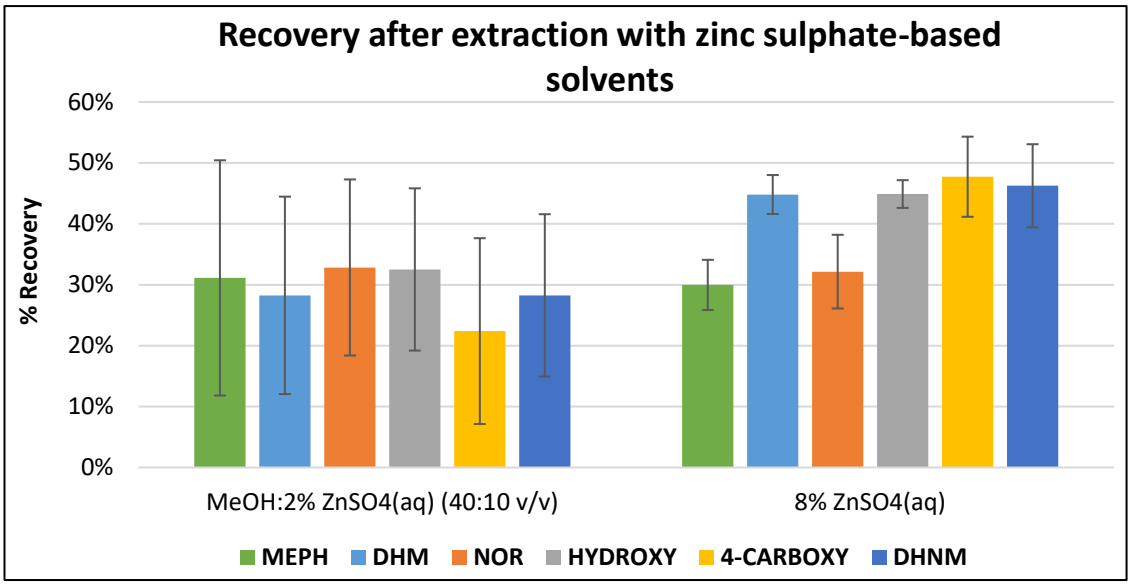


Figure 2-12. Recovery ± %CV for each analyte after an extraction with two zinc sulphate-based solvents (n=3)

2.4.7.2 Drying time

The method described in 2.4.7.1 was followed (with 0.3% FA in MeOH used as the extraction solvent) but Mitra® devices were left to dry at room temperature for 60 min, 90 min, 2 h, 2 h 30 min and 3 h. As shown in Figure 2-13, 2 h drying time at room temperature was optimal for all analytes, except for MEPH which had better recovery after 1 h of drying. The mean recovery after 2 h was 45.0 ± 0.1% for MEPH, 75.5 ± 2.4% for DHM, 57.3 ± 4.5% for NOR, 66.6 ± 5.3% for HYDROXY, 56.6 ± 7.9% for 4-CARBOXY and 82.3 ± 3.0% for DHNM.

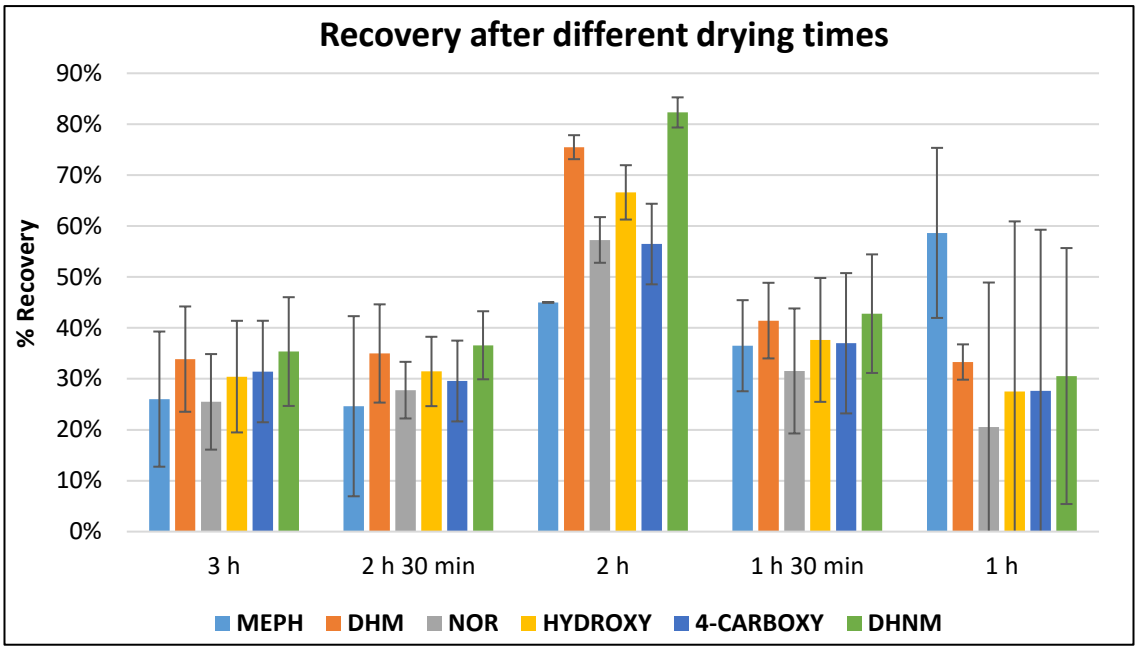


Figure 2-13. Analyte recovery ± %CV from Mitra® devices after varying drying times (n=3)

2.4.8 Fingerprint sweat

2.4.8.1 Recovery with different extraction solvent

Each finger was cleaned using an ethanol wipe and allowed to dry. A fingerprint was then deposited onto a clean circular cover slip (15 mm in diameter). Aliquots, 2 x 50 µL, of MEPH and DHM prepared in MeOH at 500 pg/mL were deposited onto the cover slips. A set of cover slips with deposited fingerprints but no analytes was also prepared. Once the solvent has evaporated, cover slips were transferred into 20 mL scintillation vials. Three hundred microliters of different extraction solvents of ranging polarities and varying pH were added to the scintillation vials. Tested solvents included ACN, MeOH, DCM, DCM:MeOH (50:50 v/v), DCM:MeOH (40:10 v/v), ethyl acetate, ACN:Water (50:50 v/v), 100 mM ammonium hydroxide:IPA (10:40 v/v), MeOH:Water (50:50 v/v), 0.2% FA in MeOH:Water (50:50 v/v), 0.2% FA in ACN:Water (50:50 v/v) and 0.2% FA in ACN:Water with varying ratios of organic to aqueous solvents. Scintillation vials were then capped and sonicated in a water bath for 7 min at 35 kHz. Extracts were transferred to 1.5 mL Eppendorf tubes and evaporated to dryness in an Eppendorf vacuum

concentrator at 45˚C. Before analysis, extracts were reconstituted with either 100 µL of 0.3% $FA_{(aq)}$ or 100 µL of a solution containing all analytes prepared in 0.3% $FA_{(aq)}$.

From the initial screen of 11 different extraction solvents (n=3), 0.2% FA in ACN:Water (1:1 v/v) gave the best recovery of 85.5 ± 0.3% for MEPH (Figure 2-14) and 96.5 ± 1.4% for DHM (Figure 2-15).

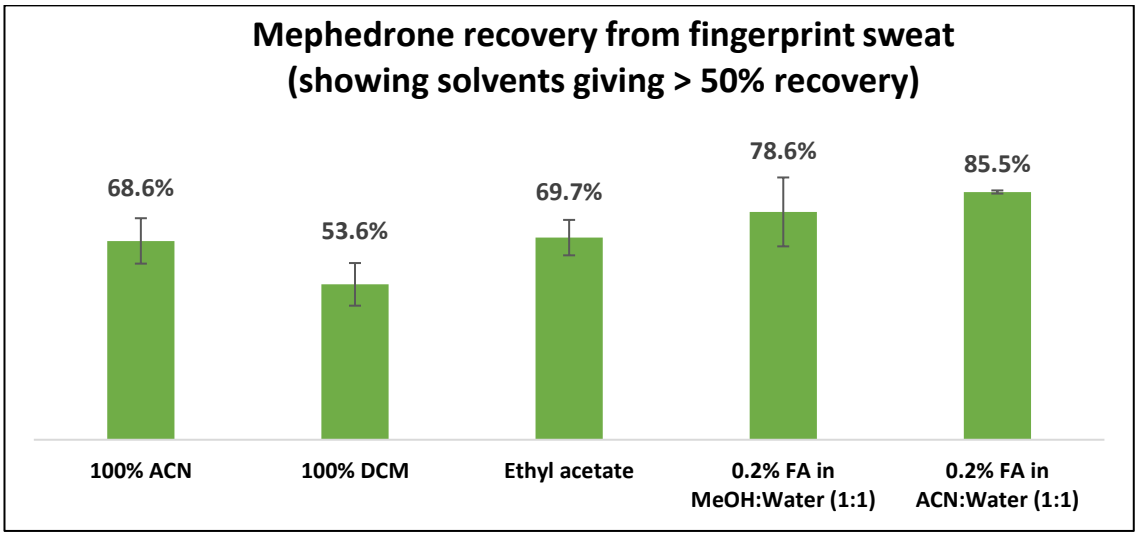


Figure 2-14. Extraction solvents giving more than 50% recovery ± %CV for MEPH from fingerprint sweat deposited on glass cover slips (n=3)

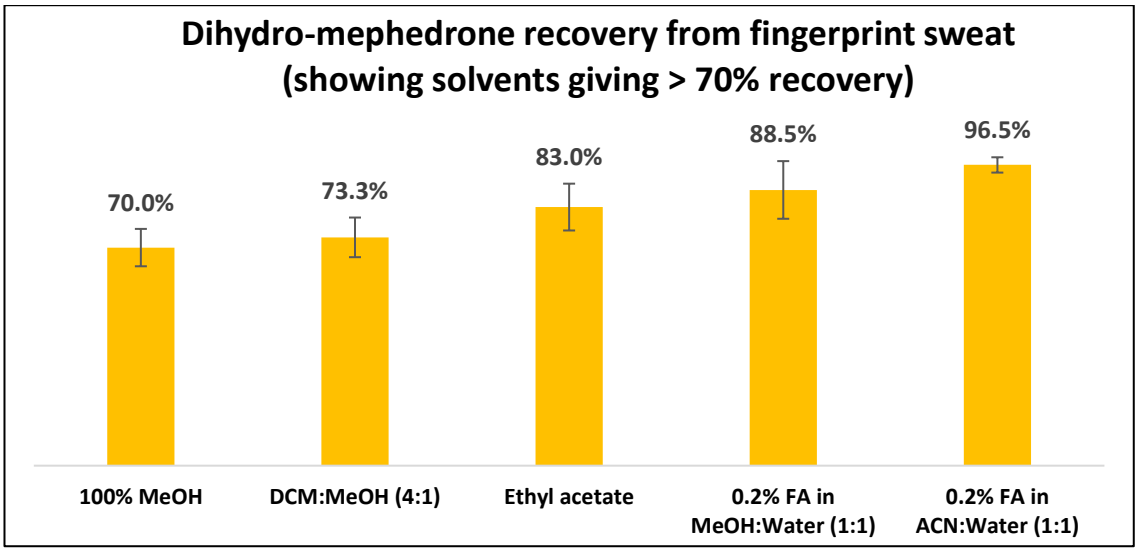


Figure 2-15. Extraction solvents giving more than 50% recovery ± %CV for DHM from fingerprint sweat deposited on glass cover slips (n=3)

Even though 0.2% FA in ACN:Water (1:1 v/v) gave the best recovery, its high water content resulted in an evaporation time of 3 h. This prompted the efforts to increase the ratio of the organic solvent which would decrease the evaporation time without impacting recovery. The following ACN:water ratios were investigated 90:10, 80:20, 70:30, 60:40, 50:50, and results are presented in Figure 2-16.

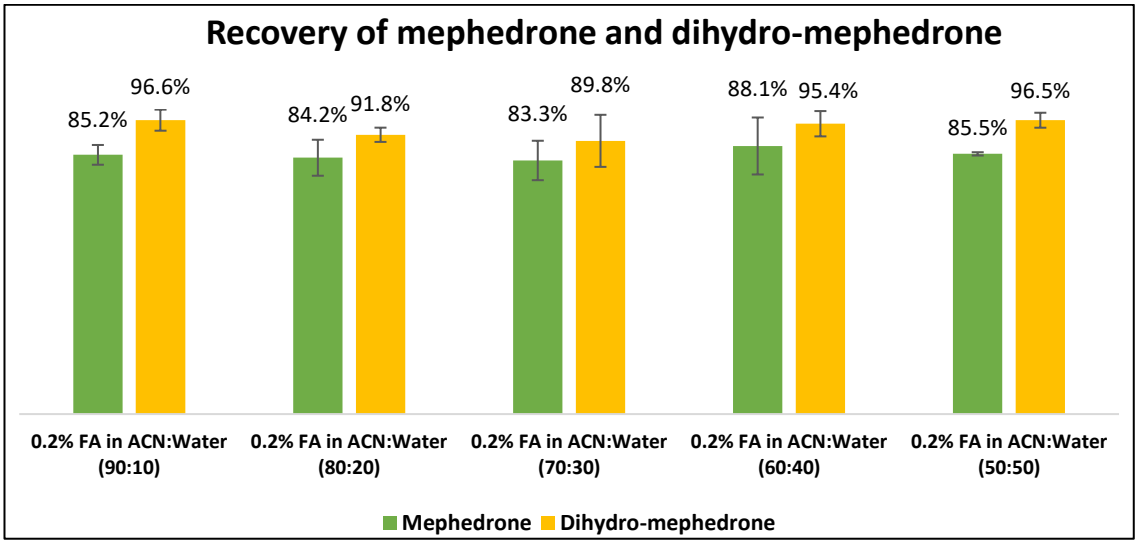


Figure 2-16. MEPH and DHM recovery ± %CV from fingerprint sweat with varying ACN:water ratios

MEPH recovery was very similar across solvents with different ACN to water ratios but 0.2% FA in ACN:Water (60:40 v/v) resulted in best recovery of 88.1 ± 4.8%. Recoveries for DHM were more variable but were greater than 89% for all tested solvents, with 0.2% FA in ACN:Water (90:10 v/v) giving the best recovery of 96.6 ± 2.5%. As it was crucial to decrease the dry-down time, 0.2% FA in ACN:Water (90:10 v/v) was chosen as the best compromise due to its high recovery and fast evaporation time of 30 min at 45°C. HYDROXY and NOR reference standards were received shortly after the completion of the recovery experiments. Their recoveries, investigated with the chosen extraction solvent, were 90.2 ± 1.9% for NOR and 68.7 ± 6.6% for HYDROXY.

ACN is a relatively polar solvent due to the electronegative nitrogen atom that attracts electrons closer to itself, resulting in an unequal electron density, which creates a dipole.

This means that there is a slightly negative charge on the nitrogen atom and a slightly positive charge on the carbon atom. The addition of formic acid to the extraction solvent changes the pH to approximately 2 which ensures that the primary amine on NOR and secondary amine on MEPH, DHM and HYDROXY are fully protonated (predicted pKa values for all are in range 7.57-9.55). The mixture of ACN and water (a polar solvent) attracts ionised analytes of interests, and thus extracts them from the fingerprint sweat.

2.4.9 Head hair

Due to time constraints, an extraction method developed and validated for amphetamines and synthetic cathinones in head hair published on the LCGC website was followed [404].

2.5 Validation procedures

Method validation, which was performed in all biological matrices, investigated the following: selectivity, linearity, inter- and intra-day precision and accuracy, limit of detection (LOD) and limit of quantification (LOQ), recovery, matrix effect, carryover, dilution integrity and stability according to the Food and Drug Administration (FDA) [405] validation guidelines and recommendations published by *Peters et al* [406].

2.5.1 Selectivity

Selectivity was assessed by analysing six blank matrix samples collected from drug-free female (n=3) and male (n=3) donors.

2.5.2 Linearity

Matrix-matched calibration curve was prepared by spiking drug-free matrix with appropriate working solutions containing mephedrone and its metabolites. Each calibration standard was required to be within ± 15% of its target concentration, except

at the lowest level of quantification (LLOQ) where ± 20% variation was allowed. The upper level of quantification (ULOQ) was defined as the highest concentration of the calibration standard. The correlation coefficient (r^2) of the curve had to be at least 0.990. A linear regression model with a weighting of 1/x was applied to all calibration curves.

2.5.3 LOD and LOQ

The LOD for each analyte in a matrix was defined as the lowest concentration where all three ions (two qualifiers and one quantifier) were present with a signal-to-noise ratio (S/N) equal to or greater than 3. The LOQ was defined as the lowest concentration at which analytes could be quantified with an acceptable precision and accuracy. The ULOQ was determined as the highest concentration of the calibration curve, which could be determined with an acceptable accuracy and precision without saturating the signal.

2.5.4 Precision and accuracy

Intra-day (n=6) and inter-day (n=3) precision and accuracy was determined by employing quality control (QC) samples spiked at low (Low), medium (Med), and high (High) concentrations. Intra-day precision was calculated using six replicates obtained on the same day which were expressed as a coefficient of variation (%CV). Accuracy was calculated by dividing the mean measured concentration at each QC level by the theoretical spiked concentration and was expressed as a percentage of the theoretical spiked concentration. Inter-day precision was evaluated for each QC level on three different days and expressed as %CV. According to the validation guidelines the mean value should be within 15% of the true value, except for the LLOQ where it should be within 20% of the true value.

2.5.5 Recovery and matrix effect

For recovery, blank matrix samples (n=6) were spiked at QC Low and QC High level and were taken through extraction. In parallel, a set of blank matrix samples (n=6) was

extracted and spiked after the evaporation step at the QC Low and QC High level. Recovery was expressed as a percentage by comparing the absolute peak areas of the samples spiked before extraction with samples spiked after extraction.

For the IS-corrected matrix effect, a set of blank matrix samples (n=6 from three female and three male donors) and a set of samples without matrix (n=6) was taken through extraction. All samples were reconstituted with a solution containing known amounts of the internal standard (IS) and analytes at QC Low and QC High levels. Matrix effect was evaluated by comparing peak area ratios in blank matrix samples spiked after extraction with peak area ratios in samples without matrix spiked after extraction.

2.5.6 Carryover

Carryover was assessed by injecting matrix blanks after the highest calibration standard. According to the validation guidelines, carryover should not exceed 20% of the LLOQ.

2.5.7 Dilution integrity

Highly concentrated samples falling outside the calibration range were diluted (see 2.6.1-2.6.8 for dilution details). Dilution integrity was assessed by preparing QC dilutions at an appropriate concentration and in an appropriate matrix outside the calibration range and diluting them into the calibration range (n=6). Precision and accuracy were required to be within ± 15% of the target concentration.

During sample analysis, QC dilutions (n=3) were prepared as described above and extracted alongside diluted samples.

2.5.8 Stability

Short-term and long-term stability as well as stability after a series of freeze-thaw cycles was assessed at QC Low and QC High levels. Samples were prepared in drug-free human

matrix and depending on the method they were stored at different conditions and for different durations of time (see 2.6.1-2.6.8 for more details). At each sampling point, one tube at each QC level was removed from storage and six aliquots were extracted alongside freshly prepared QCs. Freezer and fridge temperatures were monitored and logged daily.

2.6 Validation results

2.6.1 Whole blood (achiral method)

A method for detection and quantification of MEPH and five of its Phase I metabolites was validated in human whole blood (NaF/KOx). Validation results have been recently published [125] but they are also summarised below.

2.6.1.1 Selectivity

No interferences were observed in the extracted blank matrix.

2.6.1.2 Linearity

Mean linearity of $r^2 > 0.998$ was achieved for each analyte in all three validation runs.

2.6.1.3 LOD and LOQ

LOD and LOQ of 50 pg/mL and 200 pg/mL, respectively, was achieved for all analytes except 4-CARBOXY for which LOD was 500 pg/mL and LOQ was 2000 pg/mL, respectively. Table 2-9 shows calibration parameters for all analytes.

Table 2-9. LOD, LOQ, calibration range and calibration parameters for mephedrone and its metabolites in human whole blood (NaF/KOx)

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Range (ng/mL)	Intercept ± SD (n=3)	Slope ± SD (n=3)	r^2 ± SD (n=3)
MEPH	0.05	0.2	0.2-10	-0.013 ± 0.026	3.15 ± 0.13	0.999 ± 0.000
DHM	0.05	0.2	0.2-10	-0.032 ± 0.004	2.81 ± 0.05	0.998 ± 0.001
NOR	0.05	0.2	0.2-10	-0.015 ± 0.009	1.65 ± 0.06	0.997 ± 0.000
HYDROXY	0.05	0.2	0.2-25	-0.001 ± 0.010	1.21 ± 0.13	0.998 ± 0.001
4-CARBOXY	0.5	2	2-100	6.85 ± 5.88	27.9 ± 23.6	0.997 ± 0.002
DHNM	0.05	0.2	0.2-10	-0.004 ± 0.007	2.10 ± 0.20	0.998 ± 0.001

2.6.1.4 Precision and accuracy

Intra-day and inter-day precision and accuracy results, summarised in Table 2-10, were found to be within the acceptable limits. The intra-day inaccuracy for all metabolites was within ± 15% of the target concentration while accuracy ranged from 96.7-106% for MEPH, 91.1-109% for DHM, 89.7-97.0% for NOR, 94.3-115% for HYDROXY, 97.0-114% for 4-CARBOXY and 86.6-103% for DHNM. The intra-day imprecision was ≤ 7% and ranged from 1.44-4.33% for MEPH, 0.924-4.65% for DHM, 1.58-4.87% for NOR, 1.55-6.57% for HYDROXY, 1.36-5.97% for 4-CARBOXY, 1.52-5.13% for DHNM. Inter-day precision and accuracy results were acceptable over the validated range with %CV < 8.5% and inaccuracy within ± 9.0% of the target concentration.

*Table 2-10. Precision and accuracy at QC Low, QC Med and QC High for all analytes in human whole blood (NaF/KOx); * average value of 18 measurements over 3 days*

Analyte	True value (ng/mL)	**Mean** (ng/mL), **(%CV)**, **% accuracy**			
		Day 1 n=6	**Day 2 n=6**	**Day 3 n=6**	**Inter-day n=18 ***
MEPH	0.250	0.246 (4.33%) 98.2%	0.251 (1.44%) 101%	0.248 (3.45%) 99.3%	0.248 (3.24%) 99.3%
	1	1.00 (2.92%) 100%	0.967 (2.33%) 96.7%	1.00 (1.88%) 100%	0.988 (2.77%) 98.8%
	8	8.47 (1.72%) 106%	7.92 (2.40%) 99.0%	7.88 (1.48%) 98.6%	8.08 (3.84%) 101%
DHM	0.250	0.273 (2.68%) 109%	0.228 (2.80%) 91.1%	0.256 (4.65%) 102%	0.252 (8.27%) 101%
	1	1.02 (4.06%) 102%	0.992 (1.74%) 99.2%	1.04 (2.98%) 104%	1.02 (3.53%) 102%
	8	8.15 (0.924%) 102%	7.84 (2.22%) 98.0%	7.79 (4.05%) 97.3%	7.91 (3.28%) 98.9%
NOR	0.250	0.229 (2.55%) 91.6%	0.238 (2.97%) 95.1%	0.231 (4.87%) 92.5%	0.233 (3.77%) 93.1%
	1	0.933 (3.01%) 93.3%	0.928 (3.63%) 92.8%	0.932 (1.58%) 93.2%	0.931 (2.70%) 93.1%
	8	7.76 (3.87%) 97.0%	7.40 (4.56%) 92.5%	7.18 (3.63%) 89.7%	7.44 (5.03%) 93.0%
HYDROXY	0.250	0.236 (5.53%) 94.3%	0.267 (4.70%) 107%	0.253 (2.72%) 101%	0.252 (6.69%) 101%
	2	2.07 (3.49%) 103%	2.29 (3.89%) 115%	2.14 (2.37%) 107%	2.17 (5.42%) 108%
	20	21.1 (6.57%) 105%	23.0 (4.75%) 115%	20.1 (1.55%) 100%	21.4 (7.38%) 107%
4-CARBOXY	2.5	2.42 (1.36%) 97.0%	2.48 (3.93%) 99.1%	2.43 (4.26%) 97.0%	2.44 (3.48%) 97.7%

	20	20.7 (5.44%) 103%	21.9 (5.97%) 109%	22.9 (3.95%) 114%	21.8 (6.40%) 109%
	80	85.2 (5.40%) 107%	85.3 (3.37%) 107%	87.6 (3.60%) 109%	86.1 (4.21%) 108%
DHNM	0.250	0.228 (3.46%) 91.3%	0.238 (4.55%) 95.0%	0.239 (1.52%) 95.4%	0.235 (3.78%) 93.9%
	1	0.970 (5.13%) 97.0%	0.922 (3.72%) 92.2%	0.937 (4.99%) 93.7%	0.943 (4.92%) 94.3%
	8	8.22 (4.00%) 103%	7.49 (4.01%) 93.6%	6.93 (2.21%) 86.6%	7.55 (7.97%) 94.3%

2.6.1.5 Recovery and matrix effect

Recovery was found to be greater than 71.3% for all analytes, except for 4-CARBOXY for which recovery was 32.5 ± 6.8% at QC Low and 41.6 ± 0.5% at QC High. Reasons for such low recovery are explained in 0. Even though it is recommended for recovery to be greater than 50% [406], desired sensitivity as well as acceptable precision and accuracy were achieved for 4-CARBOXY (Table 2-10).

IS-corrected matrix effect values were within ± 17% at both QC levels, except for HYDROXY at QC High which was suppressed by 29% (Table 2-11). This may be due to the lack of matching deuterated IS which is currently not commercially available. However, assay precision and accuracy for HYDROXY at QC High were within the acceptance criteria (Table 2-10).

Table 2-11. Analyte recovery and matrix effect at QC Low and QC High in human whole blood (NaF/KOx)

Analyte	Recovery (%CV), n=6		Matrix Effect (%CV), n=6	
	QC LOW	QC HIGH	QC LOW	QC HIGH
MEPH	85.2% (1.84%)	88.3% (3.17%)	101% (8.16%)	99.0% (1.12%)
DHM	83.6% (9.97%)	84.2% (2.70%)	105% (2.87%)	98.7% (0.792%)
NOR	74.3% (3.78%)	76.6% (3.31%)	89.6% (5.89%)	91.7 % (3.25%)
HYDROXY	71.3% (4.12%)	81.4% (2.62%)	83.8% (2.58%)	71.0% (6.00%)
4-CARBOXY	32.5% (6.79%)	41.6% (0.522%)	103% (6.49%)	108% (5.99%)
DHNM	78.6% (5.70%)	79.0% (5.41%)	93.0% (4.66%)	87.4% (4.94%)

2.6.1.6 Carryover

Carryover was not observed.

2.6.1.7 Dilution integrity

Dilution integrity was assessed for whole blood samples and is presented in Table 2-12. All analytes showed acceptable precision and accuracy following 1 in 20 dilution in whole blood (NaF/KOx). %CV ranged from 5.45% to 7.98% and accuracy was within ± 15% of the target concentration.

Table 2-12. Dilution integrity (1 in 20) for MEPH and its metabolites in human whole blood (NaF/KOx)

Analyte	**1 in 20 Dilution** (n=6)			
	True value (ng/mL)	**Calculated mean** (ng/mL)	**%CV**	**% accuracy**
MEPH	1	1.01	5.47%	101%
DHM	1	0.983	5.63%	98.3%
NOR	1	0.890	5.86%	89.0%
HYDROXY	2	1.76	5.45%	87.9%
4-CARBOXY	10	11.5	7.53%	115%
DHNM	1	0.957	7.98%	95.7%

2.6.1.8 Stability

Stability was assessed after storage at +4°C and -20°C for 24 h 48 h, 4 days and 10 days. The results have been recently published (see Appendix C) [125] and are graphically summarised in Figure 2-17 - Figure 2-20. Analytes were considered unstable when they lost more than 10% of their initial concentration.

At +4°C at QC Low, DHM and DHNM were stable over the 10-day period while HYDROXY and MEPH lost 18.6 ± 5.2% and 23.4 ± 6.3%, respectively, of their initial concentration. 4-CARBOXY and NOR decreased in concentration by 48.1 ± 4.8% and 40.2 ± 6.7%, respectively, after 10 days (Figure 2-17). At QC High, DHM and DHNM were stable over the 10-day period while HYDROXY and MEPH lost 11.3 ± 3.2% and 14.2 ± 3.3%, respectively, of their initial concentration. 4-CARBOXY and NOR were most unstable and their concentration decreased by 44.6 ± 6.5% and 33.8 ± 4.2%, respectively, after 10 days (Figure 2-18).

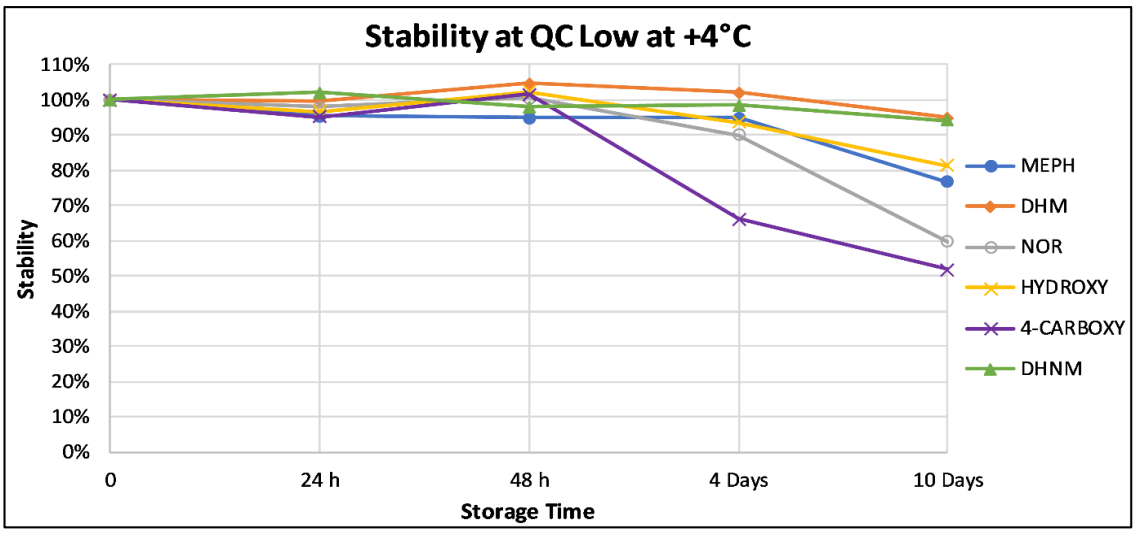


Figure 2-17. Analyte stability at QC Low in human whole blood (NaF/KOx) stored at +4°C

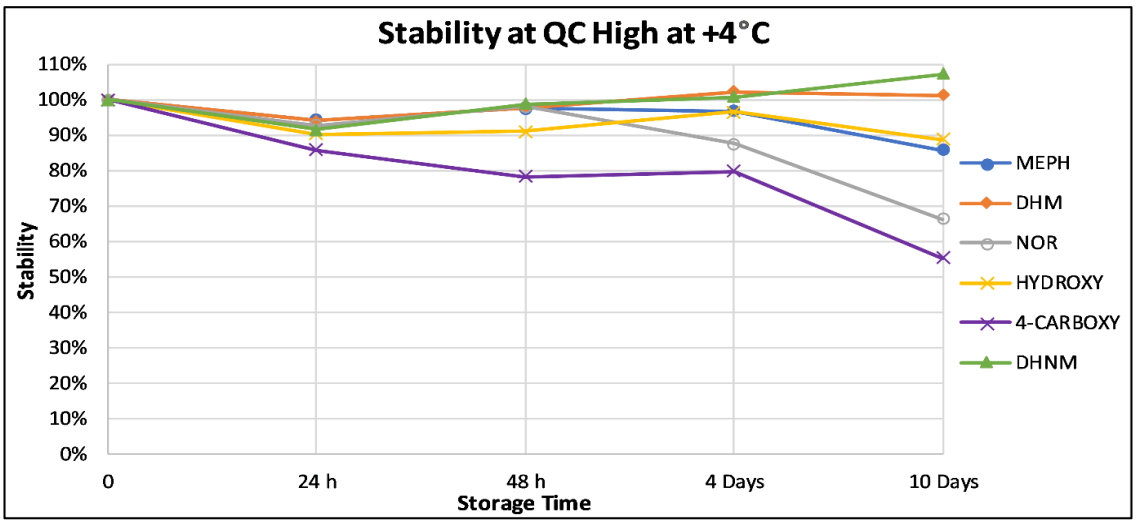


Figure 2-18. Analyte stability at QC High in human whole blood (NaF/KOx) stored at +4°C

At -20°C at QC Low, NOR and DHNM were most stable over the 10-day period while MEPH, 4-CARBOXY, HYDROXY and DHM lost 9.9 ± 2.4%, 9.6 ± 5.3%, 11.2 ± 4.8% and 12.0 ± 4.8%, respectively, of their initial concentration (Figure 2-19). At QC High, 4-CARBOXY was the most unstable and decreased in concentration by 22.6 ± 6.9% after 10 days. MEPH and DHM were stable over the 10-day period while DHNM, HYDROXY and NOR lost 7.6 ± 3.3%, 10.2 ± 2.2% and 6.6 ± 3.6%, respectively, of their initial concentration (Figure 2-20).

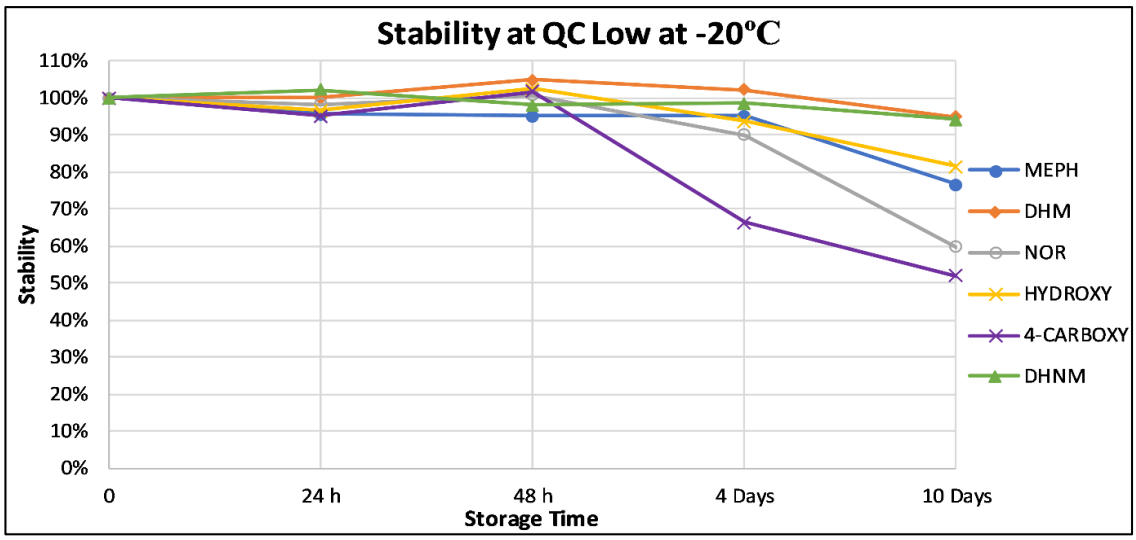


Figure 2-19. Analyte stability at QC Low in human whole blood (NaF/KOx) stored at -20°C

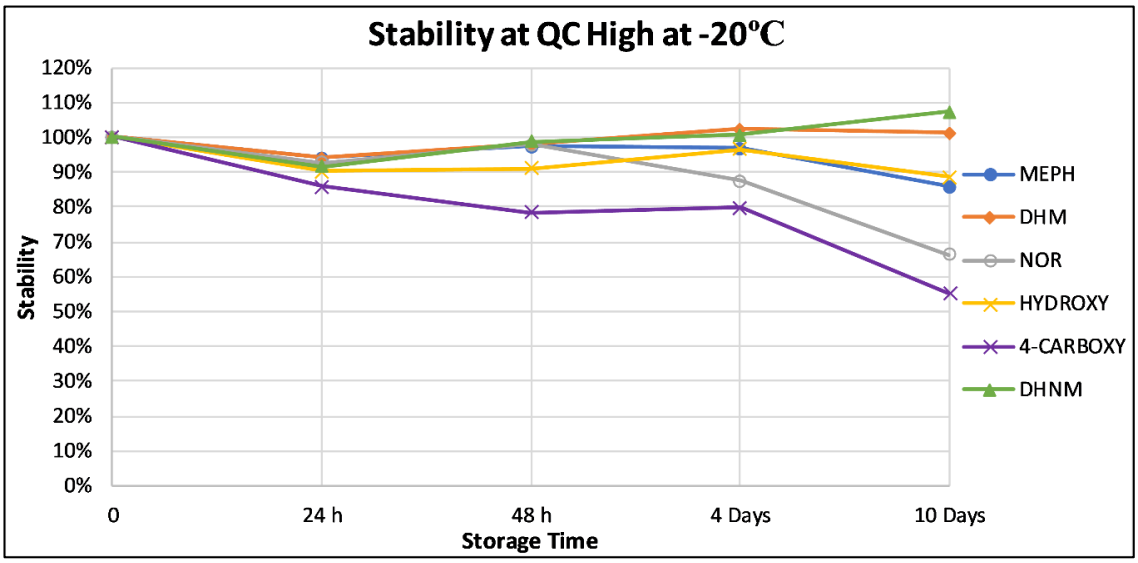


Figure 2-20. Analyte stability at QC High in human whole blood (NaF/KOx) stored at -20°C

Out of all metabolites, 4-CARBOXY was the most unstable at +4°C with significant losses observed already after 4 days (33.7 ± 6.1%) at QC Low and after 48 h (21.6 ± 4.3%) at QC High. Its stability was improved at -20°C where the highest loss of 22.6 ± 6.9% was observed after 10 days at QC High. NOR was much more stable at -20°C than 4°C where it lost 40.2 ± 6.7% at QC Low (versus no change at -20°C) and 33.8 ± 4.2% at QC High (versus 6.6 ± 3.6% at -20°C). HYDROXY was stable at -20°C but lost 18.6 ± 5.2% at +4°C

after 10 days at QC Low. DHM and DHNM remained stable at both storage conditions and concentration levels throughout the investigation with the latter showing a slight increase in its concentration after 10 days at +4°C at QC High. DHM and DHNM are the only two metabolites containing a hydroxyl group instead of a ketone at the β carbon which was previously reported to make ephedrines more stable than cathinones [121].

2.6.2 Whole blood (chiral method)

As mentioned in 0, the method was also able to separate NOR enantiomers in the same run. However, the assay (LOD of 15 ng/mL and LOQ of 60 ng/mL) was not sensitive enough to detect NOR enantiomers in whole blood samples from the administration study. As a result, method validation was only performed for (R)-mephedrone (R-MEPH) and (S)-mephedrone (S-MEPH). MEPH-d_3 was used as the IS.

2.6.2.1 Selectivity

No interferences were observed in the extracted blank matrix.

2.6.2.2 Linearity

Mean linearity of $r^2 > 0.999$ was achieved for both enantiomers in all three validation runs.

2.6.2.3 LOD and LOQ

LOD of 2 ng/mL and LOQ of 8 ng/mL was achieved for both enantiomers. Table 2-13 shows calibration parameters for both analytes.

Table 2-13. LOD, LOQ, calibration range and calibration parameters for R-MEPH and S-MEPH in human whole blood (NaF/KOx)

Analyte	**LOD** (ng/mL)	**LOQ** (ng/mL)	**Range** (ng/mL)	**Intercept ± SD** (n=3)	**Slope ± SD** (n=3)	**r^2 ± SD** (n=3)
R-MEPH	2	8	8-200	9.60 ± 1.79	13.7 ± 0.9	0.999 ± 0.001
S-MEPH	2	8	8-200	8.85 ± 1.09	13.7 ± 0.9	0.999 ± 0.001

2.6.2.4 Precision and accuracy

Intra-day and inter-day precision and accuracy results, summarised in Table 2-14, were found to be within the acceptance criteria. The intra-day inaccuracy for both enantiomers was within ± 12% of the target concentration while accuracy ranged from 101-112% for R-MEPH and 101-110% for S-MEPH. The intra-day imprecision was < 5.5% and ranged from 0.960-5.46% for R-MEPH and 1.13-4.70% for S-MEPH. Inter-day precision and accuracy results were acceptable over the validated range with %CV ≤ 4.35% and inaccuracy within ± 10% of the target concentration.

*Table 2-14. Precision and accuracy at QC Low, QC Med and QC High for R-MEPH and S-MEPH in human whole blood (NaF/KOx); * average value of 18 measurements over 3 days*

Analyte	**True value** (ng/mL)	**Mean** (ng/mL), **%CV**, **% accuracy**			
		Day 1 n=6	**Day 2 n=6**	**Day 3 n=6**	**Inter-day n=18 ***
R-MEPH	10	10.7 0.960% 107%	10.5 3.67% 105%	10.1 2.61% 101%	10.4 3.58% 104%
	40	44.3 1.74% 111%	43.0 1.24% 108%	41.0 1.21% 102%	42.7 3.54% 107%
	150	168 5.46% 112%	167 2.48% 111%	161 3.87% 107%	165 4.35% 110%
S-MEPH	10	10.4 2.83%	10.5 2.75%	10.1 1.28%	10.3 2.98%

	104%	105%	101%	103%
40	42.8 3.64% 107%	41.9 1.58% 105%	41.0 1.73% 102%	41.9 3.02% 105%
150	165 4.70% 110%	165 3.33% 110%	161 1.13% 107%	164 3.49% 109%

2.6.2.5 Recovery and matrix effect

As shown in Table 2-15, recovery for R-MEPH was found to be 82.4 ± 4.09% and 84.5 ± 7.69% at QC Low and QC High, respectively. For S-MEPH, recovery of 71.3 ± 7.83% and 85.9 ± 7.67% at QC Low and QC High, respectively, were reported. IS-corrected matrix effect values were within ± 2.5% at both QC levels.

Table 2-15. Analyte recovery and matrix effect for R-MEPH and S-MEPH at QC Low and QC High in human whole blood (NaF/KOx)

Analyte	Recovery (%CV), n=6		Matrix Effect (%CV), n=6	
	QC Low	QC High	QC Low	QC High
R-MEPH	82.4% (4.09%)	84.5% (7.69%)	102% (3.18%)	98.3% (1.81%)
S-MEPH	71.3% (7.83%)	85.9% (7.67%)	102% (7.66%)	97.5% (5.20%)

2.6.2.6 Carryover

Carryover was not observed.

2.6.2.7 Dilution integrity

Dilution integrity was not investigated because with the ULOQ of 200 ng/mL samples were anticipated to fall within the calibration range.

2.6.2.8 Stability

Stability was not assessed as samples were extracted immediately after collection.

2.6.3 Plasma

A method for detection and quantification of MEPH and five of its Phase I metabolites was validated in human plasma (NaF/KOx).

2.6.3.1 Selectivity

No interferences were observed in the extracted blank matrix.

2.6.3.2 Linearity

Mean linearity of $r^2 > 0.996$ was achieved for all analytes in all three validation runs.

2.6.3.3 LOD and LOQ

LOD of 25 pg/mL (LOQ of 100 pg/mL) for DHM, NOR and HYDROXY; 50 pg/mL (LOQ of 200 pg/mL) for DHNM; and 100 pg/mL (LOQ of 400 pg/mL) for 4-CARBOXY and MEPH were achieved in this assay. Table 2-16 shows calibration parameters for all analytes.

Table 2-16. LOD, LOQ, calibration range and calibration parameters for mephedrone and its metabolites in human plasma (NaF/KOx)

Analyte	LOD (ng/mL)	LOQ (ng/mL)	Range (ng/mL)	Intercept ± SD (n=3)	Slope ± SD (n=3)	r^2 ± SD (n=3)
MEPH	0.1	0.4	0.4-50	0.002 ± 0.004	0.274 ± 0.206	0.998 ± 0.001
DHM	0.025	0.1	0.1-50	-0.029 ± 0.019	2.30 ± 0.09	0.997 ± 0.001
NOR	0.025	0.1	0.1-50	-0.006 ± 0.005	2.31 ± 0.28	1.00 ± 0.001
HYDROXY	0.025	0.1	0.1-50	-0.043 ± 0.006	2.70 ± 0.06	0.997 ± 0.001
4-CARBOXY	0.1	0.4	0.4-50	0.011 ± 0.151	1.62 ± 0.06	0.996 ± 0.002
DHNM	0.05	0.2	0.2-50	-0.112 ± 0.060	4.64 ± 0.26	0.998 ± 0.001

2.6.3.4 Precision and accuracy

Intra-day and inter-day precision and accuracy results, summarised in Table 2-17, were found to be within the acceptance criteria. The intra-day inaccuracy was within ± 15% of the target concentration while accuracy ranged from 92.1-102% for MEPH, 97.2-106% for DHM, 89.6-101% for NOR, 85.5-104% for HYDROXY, 85.0-105% for 4-CARBOXY and 87.1-99.2% for DHNM. The intra-day precision was < 10.5% and ranged from 1.02-5.35% for MEPH, 1.10-1.90% for DHM, 0.690-5.28% for NOR, 1.44-8.28% for HYDROXY, 1.06-10.4% for 4-CARBOXY and 1.60-4.65% for DHNM. Inter-day precision and accuracy results were acceptable over the validated range with %CV < 10% and inaccuracy within ± 10.3% of the target concentration.

*Table 2-17. Precision and accuracy at QC Low, QC Med and QC High for all analytes in human plasma (NaF/KOx); * average value of 18 measurements over 3 days*

Analyte	True value (ng/mL)	Mean (ng/mL), %CV, % accuracy			
		Day 1 n=6	Day 2 n=6	Day 3 n=6	Inter-day n=18 *
MEPH	0.5	0.500 1.43% 99.4%	0.508 2.57% 102%	0.482 5.35% 96.3%	0.496 4.08% 99.1%
	5	4.79 1.36% 95.7%	4.81 1.61% 96.2%	4.77 1.70% 95.5%	4.79 1.50% 95.8%
	25	23.3 1.67% 93.3%	23.3 1.02% 93.1%	23.0 1.62% 92.1%	23.2 1.51% 92.9%
DHM	0.2	0.213 1.60% 106%	0.211 1.69% 105%	0.202 1.53% 101%	0.508 2.78% 104%
	5	5.04 1.62% 101%	4.86 1.10% 97.2%	5.07 1.43% 101%	4.99 2.34% 100%
	25	24.5 1.90% 98.1%	24.4 1.46% 97.6%	26.0 1.51% 104%	25.0 3.31% 99.9%
NOR	0.2	0.197 1.94% 98.6%	0.201 5.28% 101%	0.191 2.61% 95.5%	0.197 4.07% 98.3%
	5	4.49 3.52% 89.8%	4.57 1.28% 91.4%	4.52 1.39% 90.4%	4.53 2.28% 90.5%
	25	22.4 1.59% 89.6%	23.2 0.690% 92.7%	22.8 1.87% 91.1%	22.8 2.01% 91.1%
HYDROXY	0.2	0.194 3.21% 97.0%	0.201 1.44% 100%	0.184 2.74% 91.9%	0.192 4.40% 96.2%
	5	4.45 2.51% 88.9%	5.11 8.28% 102%	5.05 2.46% 101%	4.87 8.10% 97.4%
	25	21.4 1.96% 85.5%	26.0 4.68% 104%	24.5 4.41% 97.9%	24.5 8.20% 97.8%
4-CARBOXY	0.5	0.487 4.14% 97.4%	0.507 5.55% 101%	0.443 10.4% 88.6%	0.479 8.70% 95.8%

	5	4.25 1.06% 85.0%	4.84 8.25% 96.7%	5.26 5.98% 105%	4.89 10.0% 97.7%
	25	22.2 2.10% 88.7%	26.3 4.15% 105%	25.5 7.49% 102%	24.8 8.97% 99.2%
DHNM	0.4	0.387 2.12% 96.7%	0.397 2.86% 99.2%	0.395 2.01% 98.8%	0.393 2.26% 98.2%
	5	4.35 2.44% 87.1%	4.44 4.65% 88.8%	4.66 1.60% 93.1%	4.48 1.03% 89.7%
	25	22.5 2.65% 90.2%	22.7 3.25% 90.6%	23.1 2.54% 92.5%	22.8 2.64% 91.1%

2.6.3.5 Recovery and matrix effect

As shown in Table 2-18, recovery was found to be greater than 68.0 ± 3.65% for all analytes, except 4-CARBOXY for which recovery was 24.9 ± 6.50% at QC Low and 26.5 ± 8.11% at QC High. Reasons for such low recovery are explained in 0. Even though it is recommended for recovery to be greater than 50% [406], desired sensitivity as well as acceptable precision and accuracy were achieved for 4-CARBOXY (Table 2-17).

IS-corrected matrix effect values were within ± 15% at both QC levels for all analytes, except for HYDROXY which was suppressed by 17.1 ± 4.33% at QC Low and by 15.7 ± 4.57% at QC High. This may be due to the lack of matching deuterated IS which is currently not commercially available. However, assay precision and accuracy were within the acceptance criteria for HYDROXY (Table 2-17).

Table 2-18. Analyte recovery and matrix effect at QC Low and QC High in plasma (NaF/KOx)

Analyte	Recovery (%CV), n=6		Matrix Effect (%CV), n=6	
	QC Low	QC High	QC Low	QC High
MEPH	83.9% (4.39%)	70.0% (2.14%)	92.9% (9.92%)	96.9% (1.33%)
DHM	76.5% (1.84%)	69.8% (2.82%)	100% (3.72%)	101% (0.601%)
NOR	68.5% (1.22%)	68.0% (3.65%)	92.1% (4.61%)	90.7% (1.13%)
HYDROXY	69.8% (5.71%)	69.3% (7.34%)	82.9% (4.33%)	84.3% (4.57%)
4-CARBOXY	24.9% (6.50%)	26.5% (8.11%)	95.1% (3.92%)	92.8% (4.07%)
DHNM	73.8% (2.74%)	73.1% (4.28%)	98.6% (3.00%)	96.2% (1.14%)

2.6.3.6 Carryover

Carryover was not observed.

2.6.3.7 Dilution integrity

All analytes showed good precision and accuracy following 1 in 20 dilution in human plasma (NaF/KOx). As shown in Table 2-19, %CV ranged from 0.691-12.2% and inaccuracy was within ± 9.20% of the target concentration.

Table 2-19. Dilution integrity (1 in 20) for MEPH and its metabolites in plasma (NaF/KOx)

Analyte	**1 in 20 Dilution** (n=6)			
	True value (ng/mL)	**Calculated mean** (ng/mL)	**%CV**	**% accuracy**
MEPH	1.25	1.16	2.91%	92.9%
DHM		1.24	3.33%	98.9%
NOR		1.14	2.82%	90.8%
HYDROXY		1.24	2.00%	100%
4-CARBOXY		1.20	12.2%	96.0%
DHNM		1.20	0.691%	95.6%

2.6.3.8 Stability

Long term stability of MEPH and its metabolites in human plasma (NaF/KOx) was assessed following storage for 105 days at -20°C at QC Low and QC High. As shown in Table 2-20, analytes did not lose more than 15% of the initial concentration under the investigated conditions, except for 4-CARBOXY which lost 21.2 ± 3.77% and 27.3 ± 5.65% at QC Low and QC High, respectively.

In a separate experiment, stability samples were taken through 3 freeze-thaw (F/T) cycles from -20°C to room temperature (RT) to assess stability following sample thawing which may be required for sample re-analysis due to a failed batch or sample dilution. All analytes were stable following 3 F/T cycles, except for NOR which lost 18.6 ± 1.22% at QC High (Table 2-20).

Table 2-20. Long term and freeze-thaw stability of MEPH and its metabolites in human plasma (NaF/KOx) following storage at -20°C

Analyte	Long term stability (%CV), n=6		F/T stability (%CV), n=6	
	QC Low	QC High	QC Low	QC High
MEPH	88.1% (4.62%)	85.9% (1.98%)	101% (3.13%)	86.5% (2.06%)
DHM	104% (2.40%)	100% (2.25%)	104% (2.82%)	101% (1.70%)
NOR	102% (1.89%)	86.1% (1.85%)	97.5% (3.05%)	81.4% (1.22%)
HYDROXY	96.9% (4.07%)	91.4% (4.85%)	96.9% (4.07%)	92.7% (1.96%)
4-CARBOXY	78.8% (3.77%)	72.7% (5.65%)	89.0% (4.36%)	91.2% (3.34%)
DHNM	99.2% (0.559%)	96.8% (1.55%)	97.8% (2.92%)	94.7% (2.72%)

2.6.4 Urine

A method for detection and quantification of MEPH and five of its Phase I metabolites was validated in human urine.

2.6.4.1 Selectivity

No interferences were observed in the extracted blank matrix.

2.6.4.2 Linearity

Mean linearity of $r^2 > 0.996$ was achieved for all analytes in all three validation runs.

2.6.4.3 LOD and LOQ

LOD of 0.4 ng/mL (LOQ of 1.6 ng/mL) for MEPH, HYDROXY, 4-CARBOXY; 0.15 ng/mL (LOQ of 0.6 ng/mL) for DHM, NOR; and 0.08 ng/mL (LOQ of 0.6 ng/mL) for DHNM were achieved in this assay. Table 2-21 shows calibration parameters for all analytes.

Table 2-21. LOD, LOQ, calibration range and calibration parameters for mephedrone and its metabolites in human urine

Analyte	**LOD** (ng/mL)	**LOQ** (ng/mL)	**Range** (ng/mL)	**Intercept ± SD** (n=3)	**Slope ± SD** (n=3)	**r^2 ± SD** (n=3)
MEPH	0.4	1.6	1.6-50	0.353 ± 0.670	1.97 ± 0.04	0.998 ± 0.001
DHM	0.15	0.6	0.6-50	-0.483 ± 0.363	13.8 ± 0.5	1.00 ± 0.00
NOR	0.15	0.6	0.6-50	-0.407 ± 0.246	13.0 ± 1.0	0.999 ± 0.001
HYDROXY	0.4	1.6	1.6-50	0.068 ± 0.455	4.66 ± 0.47	0.999 ± 0.000
4-CARBOXY	0.4	1.6	1.6-50	-0.844 ± 0.871	4.81 ± 0.57	0.996 ± 0.001
DHNM	0.08	0.6	0.6-50	-0.973 ± 0.214	16.2 ± 1.9	0.999 ± 0.000

2.6.4.4 Precision and accuracy

Intra-day and inter-day precision and accuracy results, summarised in Table 2-22, were found to be within the acceptance criteria. The intra-day inaccuracy was within ± 11.3%

of the target concentration while accuracy ranged from 94.7-104% for MEPH, 98.5-107% for DHM, 90.8-102% for NOR, 89.7-104% for HYDROXY, 88.7-103% for 4-CARBOXY and 94.3-102% for DHNM. The intra-day imprecision was ≤ 9.39% and ranged from 1.65-5.16% for MEPH, 1.44-6.61% for DHM, 0.978-4.18% for NOR, 1.89-5.23% for HYDROXY, 4.15-9.39% for 4-CARBOXY and 0.956-6.21% for DHNM. Inter-day precision and accuracy results were acceptable over the validated range with %CV ≤ 7.63% and inaccuracy within ± 7.50% of the target concentration.

*Table 2-22. Precision and accuracy at QC Low, QC Med and QC High for all analytes in human urine; * average value of 18 measurements over 3 days*

Analyte	**True value** (ng/mL)	**Mean** (ng/mL), **%CV**, **% accuracy**			
		Day 1 n=6	**Day 2 n=6**	**Day 3 n=6**	**Inter-day n=18 ***
MEPH	2	1.89 3.95% 94.7%	2.02 4.76% 101%	2.01 4.50% 100%	1.97 5.14% 98.7%
	10	9.83 3.34% 98.3%	9.95 5.16% 99.5%	10.4 1.65% 104%	10.0 4.11% 100%
	40	38.0 3.60% 94.9%	38.2 2.22% 95.4%	39.5 1.91% 98.8%	38.6 3.12% 96.4%
DHM	1	1.03 5.15% 103%	1.07 1.44% 107%	1.02 3.62% 102%	1.04 4.02% 104%
	10	10.3 4.35% 103%	10.4 3.79% 104%	10.6 6.61% 106%	10.4 4.89% 104%
	40	39.5 6.27% 98.8%	41.2 3.05% 103%	39.4 2.13% 98.5%	40.0 4.48% 100%
NOR	1	0.908 2.81% 90.8%	0.968 2.16% 96.8%	0.974 3.00% 97.4%	0.950 4.09% 95.0%
	10	10.1 4.18% 101%	10.2 1.65% 102%	9.70 0.978% 97.0%	9.99 3.26% 99.9%
	40	38.6	40.0	40.1	1.23

		3.79% 96.5%	1.66% 100%	2.38% 100%	3.12% 99.0%
HYDROXY	2	2.01 4.13% 101%	1.91 2.98% 95.4%	1.92 2.04% 95.9%	1.95 3.87% 97.3%
	10	10.1 3.31% 101%	10.4 3.50% 104%	9.62 2.68% 96.2%	10.1 4.51% 101%
	40	38.7 5.23% 96.7%	41.0 1.89% 102%	35.9 2.82% 89.7%	38.5 6.47% 96.3%
4-CARBOXY	2	2.02 8.71% 101%	1.88 4.59% 94.2%	2.06 5.59% 103%	1.99 7.32% 99.4%
	10	9.39 4.46% 93.9%	9.98 9.39% 99.8%	9.67 4.15% 96.7%	9.68 6.69% 96.8%
	40	36.5 6.76% 91.1%	39.0 6.14% 97.5%	35.5 7.61% 88.7%	37.0 7.63% 92.5%
DHNM	1	0.943 5.48% 94.3%	0.985 2.61% 98.5%	0.963 3.11% 96.3%	0.964 0.920% 96.4%
	10	10.0 4.29% 100%	10.2 5.31% 102%	9.62 2.68% 96.2%	10.1 0.461% 101%
	40	39.5 6.21% 98.7%	406 1.16% 101%	38.6 0.956% 96.5%	39.6 1.52% 98.8%

2.6.4.5 Recovery and matrix effect

As shown in Table 2-23, recovery was found to be greater than 74.7 ± 3.79% for all analytes, except 4-CARBOXY for which recovery was 35.8 ± 7.18% at QC Low and 34.3 ± 6.30% at QC High. Reasons for such low recovery are explained in 0. Even though it is recommended for recovery to be greater than 50% [406], desired sensitivity as well as acceptable precision and accuracy were achieved for 4-CARBOXY (Table 2-22).

IS-corrected matrix effect values were within ± 20.6% at both QC levels, except for HYDROXY which was suppressed by 34.4 ± 7.37% at QC Low and by 32.6 ± 4.57% at QC High (Table 2-23). This may be due to the lack of matching deuterated IS which is currently not commercially available. However, assay precision and accuracy for HYDROXY at QC Low and QC High were within the acceptable limits (Table 2-22).

Table 2-23. Analyte recovery and matrix effect at QC Low and QC High in human urine

Analyte	Recovery (%CV), n=6		Matrix Effect (%CV), n=6	
	QC Low	QC High	QC Low	QC High
MEPH	91.7% (10.4%)	82.6% (5.56%)	98.3% (5.00%)	95.5% (0.761%)
DHM	79.5% (6.01%)	82.4% (5.27%)	101% (0.984%)	98.6% (0.424%)
NOR	74.7% (3.79%)	79.0% (5.36%)	93.2% (1.23%)	90.9% (0.489%)
HYDROXY	84.3% (6.09%)	87.2% (6.02%)	65.6% (7.37%)	67.4% (4.57%)
4-CARBOXY	35.8% (7.18%)	34.3% (6.30%)	80.3% (6.90%)	79.4% (4.98%)
DHNM	90.3% (5.41%)	97.7% (5.48%)	91.4% (2.97%)	88.6% (1.05%)

2.6.4.6 Carryover

Carryover was not observed.

2.6.4.7 Dilution integrity

All analytes showed good precision and accuracy following 1 in 100 and 1 in 1000 dilutions in human urine. For 1 in 100 dilution, %CV ranged from 4.44-11.0% and inaccuracy was within ± 6.0% of the target concentration (Table 2-24). For 1 in 1000 dilution, %CV ranged from 2.43-6.57% and inaccuracy was within ± 7.0% of the target concentration (Table 2-25).

Table 2-24. Dilution integrity (1 in 100) for MEPH and its metabolites in human urine

Analyte	**1 in 100 Dilution** (n=6)			
	True value (ng/mL)	**Calculated mean** (ng/mL)	**%CV**	**% accuracy**
MEPH	10	9.98	4.44%	99.8%
DHM		10.0	6.33%	100%
NOR		9.75	6.35%	97.5%
HYDROXY		10.2	11.0%	102%
4-CARBOXY		10.6	10.7%	106%
DHNM		9.82	8.60%	98.2%

Table 2-25. Dilution integrity (1 in 1000) for MEPH and its metabolites in human urine

Analyte	**1 in 1000 Dilution** (n=6)			
	True value (ng/mL)	**Calculated mean** (ng/mL)	**%CV**	**% accuracy**
MEPH	10	9.90	2.43%	99.0%
DHM		10.3	3.83%	103%
NOR		9.30	5.15%	93.0%
HYDROXY		9.44	3.50%	94.4%
4-CARBOXY		9.73	6.57%	97.3%
DHNM		9.57	3.90%	95.7%

2.6.4.8 Stability

Long term stability of MEPH and its metabolites in human urine was assessed following storage for 105 days at -20°C at QC Low and QC High. As shown in Table 2-26, all analytes were within ± 15% of their initial concentration under the investigated conditions, except for DHNM at QC Low as well as HYDROXY and 4-CARBOXY at QC High which lost 61.2 ± 2.90%, 33.4 ± 4.07% and 43.2 ± 8.89%, respectively.

In a separate experiment, stability samples were taken through 6 F/T cycles from -20°C to RT to assess stability following sample thawing which may be required for sample re-analysis due to a failed batch or sample dilution. All analytes were stable following 6 F/T cycles, except 4-CARBOXY at QC High and DHNM at QC Low, which lost 17.2 ± 5.53% and 18.8 ± 5.25%, respectively.

Table 2-26. Long term and freeze-thaw stability of MEPH and its metabolites in human urine following storage at -20°C

Analyte	Long term stability (%CV), n=6		F/T stability (%CV), n=6	
	QC Low	QC High	QC Low	QC High
MEPH	101% (2.54%)	99.3% (1.26%)	102% (2.57%)	93.8% (0.783%)
DHM	92.0% (1.69%)	102% (0.731%)	100% (1.80%)	98.5% (1.40%)
NOR	102% (2.41%)	90.9% (1.21%)	97.5% (2.01%)	86.4% (1.87%)
HYDROXY	89.3% (2.74%)	66.6% (4.07%)	102% (3.19%)	90.2% (3.42%)
4-CARBOXY	94.6% (1.51%)	56.8% (8.89%)	103% (6.90%)	82.8% (5.53%)
DHNM	38.8% (2.90%)	90.3% (1.09%)	81.2% (5.25%)	97.9% (1.06%)

2.6.5 Oral fluid

A method for detection and quantification of MEPH and three of its Phase I metabolites (DHM, NOR and DHNM) was validated in oral fluid.

2.6.5.1 Selectivity

No interferences were observed in the extracted blank matrix.

2.6.5.2 Linearity

Mean linearity of $r^2 > 0.998$ was achieved for all analytes in all three validation runs.

2.6.5.3 LOD and LOQ

LOD of 0.1 ng/mL (LOQ of 0.5 ng/mL) for MEPH; 0.5 ng/mL (LOQ of 2 ng/mL) for DHM; and 1 ng/mL (LOQ of 4 ng/mL) for NOR and DHNM were achieved in this assay. Table 2-27 shows calibration parameters for all analytes.

Table 2-27. LOD, LOQ, calibration range and calibration parameters for mephedrone and its metabolites in oral fluid

Analyte	**LOD** (ng/mL)	**LOQ** (ng/mL)	**Range** (ng/mL)	**Intercept ± SD** (n=3)	**Slope ± SD** (n=3)	**r^2 ± SD** (n=3)
MEPH	0.1	0.5	0.5-500	0.103 ± 0.138	0.064 ± 0.008	0.998 ± 0.002
DHM	0.5	2	2-500	0.157 ± 0.046	0.046 ± 0.004	0.999 ± 0.001
NOR	1	4	4-500	-0.251 ± 0.053	0.039 ± 0.008	0.999 ± 0.001
DHNM	1	4	4-500	-0.076 ± 0.088	0.013 ± 0.008	0.998 ± 0.003

2.6.5.4 Precision and accuracy

Intra-day and inter-day precision and accuracy results, summarised in Table 2-28, were found to be within the acceptance criteria. The intra-day inaccuracy was within ± 13% of the target concentration while accuracy ranged from 95.1-108% for MEPH, 87.8-110% for DHM, 92.1-106% for NOR, and 93.8-113% for DHNM. The intra-day imprecision was ≤ 10% and ranged from 2.50-9.95% for MEPH, 0.363-2.23% for DHM, 0.451-9.62% for NOR and 0.737-4.31% for DHNM. Inter-day precision and accuracy results were acceptable over the validated range with %CV ≤ 10.8% and inaccuracy within ± 5.6% of the target concentration.

*Table 2-28. Precision and accuracy at QC Low, QC Med and QC High for all analytes in oral fluid; * average value of 18 measurements over 3 days*

Analyte	True value (ng/mL)	Mean (ng/mL), %CV, % accuracy			
		Day 1 n=6	Day 2 n=6	Day 3 n=6	Inter-day n=18 *
MEPH	0.5	0.530 9.95% 106%	0.513 4.38% 103%	0.541 5.60% 108%	0.527 7.07% 105%
	50	49.7 2.50% 99.3%	54.0 5.77% 108%	49.0 3.83% 98.1%	50.9 6.05% 102%
	250	238 3.54% 95.1%	241 3.93% 96.3%	239 9.19% 95.7%	13.8 5.78% 95.7%
DHM	4	4.10 0.363% 103%	4.10 0.543% 103%	3.98 0.656% 100%	4.06 1.53% 102%
	50	43.9 2.23% 87.8%	45.2 1.33% 90.4%	55.0 1.69% 110%	48.0 10.8% 96.1%
	250	236 1.80% 94.2%	235 2.20% 93.9%	257 1.91% 103%	242 4.69% 96.9%
NOR	7	6.62 0.451% 94.6%	6.62 0.595% 101%	6.59 0.479% 94.1%	6.61 0.527% 94.4%
	50	46.3 2.65% 92.6%	50.4 3.74% 101%	53.2 9.62% 106%	49.8 8.15% 99.5%
	250	247 3.65% 99.0%	248 3.30% 99.3%	230 6.77% 92.1%	242 5.68% 96.8%
DHNM	5	4.79 0.737% 95.9%	4.69 4.31% 93.9%	5.64 0.790% 113%	5.01 0.177% 100%
	50	47.3 3.03% 94.7%	49.1 4.24% 98.2%	53.9 2.94% 108%	50.1 0.093% 100%
	250	243 3.06% 97.3%	240 3.49% 95.9%	234 3.36% 93.8%	239 0.251% 95.7%

2.6.5.5 Recovery and matrix effect

As shown in Table 2-29, recovery at 5 ng/mL was found to be greater than 62.6 ± 6.98% for all analytes. IS-corrected matrix effect values were within ± 9% at QC Low and within ± 4% at QC High, demonstrating only small analyte signal enhancement/suppression.

Table 2-29. Analyte recovery at 5 ng/mL and matrix effect at QC Low and QC High in oral fluid

Analyte	Recovery (%CV), n=6	Matrix Effect (%CV), n=6	
	5 ng/mL	QC Low	QC High
MEPH	78.2% (3.36%)	100% (2.45%)	104% (1.88%)
DHM	62.6% (6.98%)	100% (1.24%)	100% (1.49%)
NOR	73.0% (4.45%)	109% (1.84%)	100% (3.11%)
DHNM	62.7% (6.24%)	105% (4.14%)	98.7% (2.35%)

2.6.5.6 Carryover

Carryover was not observed.

2.6.5.7 Dilution integrity

Dilution integrity was not assessed.

2.6.6 Dried blood spots

A method for detection and quantification of MEPH and five of its Phase I metabolites was validated in human dried blood spots (DBS) collected by the Mitra® device.

2.6.6.1 Selectivity

No interferences were observed in the extracted blank matrix.

2.6.6.2 Linearity

Mean linearity of $r^2 > 0.997$ was achieved for all analytes in all three validation runs.

2.6.6.3 LOD and LOQ

LOD of 2 ng/mL (LOQ of 8 ng/mL) for MEPH; 0.5 ng/mL (LOQ of 2 ng/mL) for DHM, HYDROXY, DHNM; and 1 ng/mL (LOQ of 4 ng/mL) for NOR, 4-CARBOXY were achieved in this assay. Table 2-30 shows calibration parameters for all analytes.

Table 2-30. LOD, LOQ, calibration range and calibration parameters for mephedrone and its metabolites in dried blood spots

Analyte	**LOD** (ng/mL)	**LOQ** (ng/mL)	**Range** (ng/mL)	**Intercept ± SD** (n=3)	**Slope ± SD** (n=3)	**r^2 ± SD** (n=3)
MEPH	2	8	8-500	0.004 ± 0.001	0.036 ± 0.003	0.998 ± 0.002
DHM	0.5	2	2-500	-0.000 ± 0.008	0.035 ± 0.002	0.997 ± 0.002
NOR	1	4	4-500	0.000 ± 0.000	0.022 ± 0.003	0.998 ± 0.001
HYDROXY	0.5	2	2-500	0.003 ± 0.002	0.016 ± 0.003	0.998 ± 0.002
4-CARBOXY	1	4	4-500	0.008 ± 0.002	0.021 ± 0.008	0.998 ± 0.001
DHNM	0.5	2	2-500	-0.006 ± 0.000	0.041 ± 0.003	0.998 ± 0.001

2.6.6.4 Precision and accuracy

Intra-day and inter-day precision and accuracy results, summarised in Table 2-31, were found to be within the acceptance criteria. The intra-day inaccuracy was within ± 14% of the target concentration while accuracy ranged from 95.1-106% for MEPH, 99.0-113% for DHM, 87.1-104% for NOR, 99.2-114% for HYDROXY, 92.9-114% for 4-CARBOXY and 94.6-104% for DHNM. The intra-day imprecision was ≤ 14.3% and ranged from

2.20-10.9% for MEPH, 2.26-5.72% for DHM, 2.14-11.8% for NOR, 2.95-13.7% for HYDROXY, 2.76-14.3% for 4-CARBOXY and 1.55-5.13% for DHNM. Inter-day precision and accuracy results were acceptable over the validated range with %CV ≤ 11.4% and inaccuracy within ± 12% of the target concentration.

*Table 2-31. Precision and accuracy at QC Low, QC Med and QC High for all analytes in dried blood spots; * average value of 18 measurements over 3 days*

Analyte	**True value** (ng/mL)	**Mean** (ng/mL), **%CV**, **% accuracy**			
		Day 1 n=6	**Day 2 n=6**	**Day 3 n=6**	**Inter-day n=18 ***
MEPH	10	10.4 4.40% 104%	10.1 4.68% 101%	9.77 4.80% 97.7%	10.1 5.05% 101%
	50	53.0 2.20% 106%	51.9 10.9% 104%	50.6 5.09% 101%	51.8 6.93% 104%
	400	410 3.95% 102%	396 4.21% 99.1%	381 5.23% 95.1%	395 5.21% 98.9%
DHM	4	4.14 3.90% 104%	3.96 4.03% 99.0%	4.30 2.89% 107%	4.13 4.82% 103%
	50	56.6 2.26% 113%	54.1 3.24% 108%	54.5 3.05% 109%	55.1 3.40% 110%
	400	431 4.08% 108%	410 3.97% 103%	398 5.72% 99.6%	4.92 5.98% 98.3%
NOR	5	5.17 2.14% 103%	4.99 2.17% 100%	4.60 4.85% 91.9%	48.5 11.3% 97.0%
	50	49.9 2.47% 100%	52.1 11.8% 104%	43.6 8.86% 87.1%	48.5 11.3% 97.0%
	400	407 3.34% 102%	413 3.96% 103%	363 4.45% 90.8%	394 6.85% 98.6%
HYDROXY	4	3.97 4.38% 99.2%	3.98 10.3% 99.4%	4.05 13.4% 101%	4.00 9.56% 100%

	50	55.7 2.95% 111%	55.2 7.99% 110%	56.9 10.9% 114%	55.9 7.66% 112%
	400	413 3.64% 103%	442 8.77% 111%	397 13.7% 99.3%	417 10.0% 104%
4-CARBOXY	5	4.82 3.60% 96.5%	4.64 9.36% 92.9%	5.07 11.3% 101%	4.86 8.64% 97.2%
	50	55.7 2.76% 111%	49.7 10.8% 99.4%	57.2 14.3% 114%	54.0 11.4% 108%
	400	427 4.00% 107%	450 8.12% 112%	392 13.0% 98.0%	427 9.36% 107%
DHNM	4	4.04 2.45% 101%	3.78 2.95% 94.6%	3.80 3.07% 94.9%	3.87 4.10% 96.8%
	50	51.3 1.55% 103%	49.8 3.48% 99.5%	48.3 4.31% 96.6%	49.8 4.01% 99.6%
	400	418 3.20% 104%	401 3.87% 100%	381 5.13% 95.2%	400 5.48% 100%

By collecting an accurate volume of matrix, Mitra® devices overcome the issues associated with the blood haematocrit (Hct) [274]. Therefore, only 3 replicates at low (20% Hct) and high (60% Hct) Hct values were tested for accuracy and precision. As shown in Table 2-32, inaccuracy was within ± 11% at QC Low, ± 14% at QC Med and ± 15% at QC High of the target concentration across both Hct levels. Imprecision was ≤ 12.9% and ranged from 0.199-8.45% at QC Low, 1.60-8.37% at QC Med and 0.400-12.9% at QC High at both Hct levels.

Table 2-32. Precision and accuracy at 20% and 60% Hct at QC Low, QC Med and QC High in dried blood spots

Analyte	**Mean** (ng/mL), **%CV**, **% accuracy**					
	QC LOW Hct 20%	**QC LOW Hct 60%**	**QC MED Hct 20%**	**QC MED Hct 60%**	**QC HIGH Hct 20%**	**QC HIGH Hct 60%**
MEPH	9.94 0.695% 99.4%	10.1 2.13% 101%	51.6 1.60% 103%	54.4 5.23% 109%	352 2.47% 87.9%	462 3.05% 115%
DHM	4.16 3.58% 104%	4.28 4.37% 107%	54.2 3.72% 108%	49.5 2.36% 99.1%	366 1.37% 91.6%	357 2.57% 89.4%
NOR	4.95 3.35% 99.1%	4.90 8.45% 97.9%	45.0 5.14% 90.0%	48.4 6.13% 96.8%	343 0.454% 85.8%	455 0.400% 114%
HYDROXY	3.56 3.35% 89.0%	3.98 5.80% 99.5%	52.9 10.1% 106%	56.9 8.37% 114%	350 2.98% 87.5%	444 9.57% 111%
4-CARBOXY	4.72 4.54% 94.5%	4.98 0.199% 100%	57.0 4.17% 114%	53.6 9.70% 107%	391 6.74% 97.8%	425 12.9% 106%
DHNM	4.09 1.72% 102%	3.87 5.75% 96.8%	51.2 3.43% 102%	51.2 1.93% 102%	371 1.34% 92.7%	458 0.889% 114%

2.6.6.5 Recovery and matrix effect

As shown in Table 2-33, recovery at 10 ng/mL was found to be greater than 81.5 ± 4.53% for all analytes, except 4-CARBOXY for which recovery was 69.5 ± 4.51%. IS-corrected matrix effect values were within ± 17% at both QC levels, with NOR and DHNM being enhanced by 16 ± 7.73% and 17 ± 6.56% at QC High, respectively.

Table 2-33. Analyte recovery at 10 ng/mL and matrix effect at QC Low and QC High in DBS

Analyte	Recovery (%CV), n=6	Matrix Effect (%CV), n=6	
	10 ng/mL	QC Low	QC High
MEPH	81.5% (4.53%)	99.4% (2.69%)	85.4% (4.85%)
DHM	97.2% (8.31%)	108% (10.8%)	104% (6.88%)
NOR	84.9% (1.25%)	111% (4.50%)	116% (7.73%)
HYDROXY	90.8% (1.26%)	86.6% (3.51%)	102% (6.34%)
4-CARBOXY	69.5% (4.51%)	94.1% (2.27%)	113% (4.30%)
DHNM	101% (1.85%)	105% (2.29%)	117% (6.56%)

2.6.6.6 Carryover

Carryover was observed for mephedrone. In order to tackle this problem, three methanol blanks followed by a matrix blank were run after the highest calibration standard and after samples suspected of high analyte concentration. Carryover was not seen for mephedrone in whole blood/plasma because the ULOQ in these two matrices was 50 times lower than in DBS.

2.6.6.7 Dilution integrity

Before sample analysis commenced, mephedrone had been anticipated to be the only analyte which would be found at concentrations outside the calibration range. Therefore, dilution integrity was only assessed for mephedrone. Due to the nature of DBS collection, dilution was impractical at the beginning of an extraction as it would normally be the case with blood or urine. Moreover, only one sample was collected at each timepoint during the administration study making repeated analysis impossible. Therefore, dilution was performed after sample reconstitution, when an appropriate volume of the reconstituted sample was diluted 1 in 10 in the reconstitution solvent. As a result, internal standard was also diluted 1 in 10. In order to still be able to detect the

internal standard after 1 in 10 dilution, concentration of the internal standard used in this method was increased to 250 ng/mL.

Good precision (7.77%) and accuracy (107%) was achieved for mephedrone following 1 in 10 dilution (Table 2-34).

Table 2-34. Dilution integrity (1 in 10) for mephedrone in dried blood spots

Analyte	**1 in 10 Dilution** (n=6)			
	True value (ng/mL)	**Calculated mean** (ng/mL)	**%CV**	**% accuracy**
MEPH	100	107	7.77%	107%

2.6.6.8 Stability

Short term stability of mephedrone and its metabolites in DBS collected by the Mitra® device was assessed after storage in desiccated conditions for 10 days and 1 month at RT and +4°C (stability after storage at -20°C was not assessed due to time constraints) at QC Low and QC High.

As shown in Table 2-35, analytes lost more than 15% of their initial concentration after storage, except for DHM and DHNM at both QC levels. HYDROXY and 4-CARBOXY showed the greatest losses with the analytes not being detectable at QC Low after 10 days of storage at +4°C and RT. As shown in Table 2-36, further losses were observed after 1 month of storage. DHM and DHNM considered stable under the investigated conditions after 10 days, showed instability greater than 15% of their initial concentration at QC Low. HYDROXY and 4-CARBOXY remained undetectable at QC Low and suffered losses of up to 99.9% at QC High. Even though one of the advantages of DBS is the ability to transport and store these samples in ambient conditions, this would not be recommended for mephedrone and its metabolites. The results demonstrate

that DBS samples should not be stored at +4°C or at RT due to significant analyte instability even when desiccated.

Table 2-35. 10-day stability of mephedrone and its metabolites in dried blood spots following storage at RT and +4°C; ND – not detected

Analyte	Storage at +4°C (%CV), n=6		Storage at RT (%CV), n=6	
	QC Low	QC High	QC Low	QC High
MEPH	11.8% (13.2%)	10.4% (5.47%)	5.88% (14.7%)	5.44% (20.0%)
DHM	89.1% (5.96%)	93.3% (9.29%)	91.1% (15.8%)	83.4% (3.81%)
NOR	32.5% (8.63%)	30.7% (4.46%)	33.4% (14.9%)	25.3% (5.75%)
HYDROXY	ND	0.566% (6.36%)	ND	0.957% (12.1%)
4-CARBOXY	ND	0.128% (9.33%)	ND	0.667% (18.6%)
DHNM	97.4% (5.53%)	101% (6.24%)	99.2% (8.06%)	89.3% (4.14%)

Table 2-36. 1-month stability of mephedrone and its metabolites in dried blood spots following storage at RT and +4°C; ND – not detected

Analyte	Storage at +4°C (%CV), n=6		Storage at RT (%CV), n=6	
	QC Low	QC High	QC Low	QC High
MEPH	13.0% (12.4%)	6.45% (9.42%)	5.68% (6.14%)	2.09% (13.0%)
DHM	81.5% (13.2%)	96.7% (6.92%)	61.1% (12.7%)	83.4% (5.52%)
NOR	25.5% (11.6%)	24.6% (6.57%)	13.9% (14.3%)	16.5% (13.1%)
HYDROXY	ND	0.250% (6.96%)	ND	0.717% (8.93%)
4-CARBOXY	ND	0.069% (16.6%)	ND	0.448% (9.13%)
DHNM	81.4% (3.34%)	102% (4.44%)	84.3% (6.45%)	89.5% (9.15%)

2.6.7 Fingerprint sweat

A method for detection and quantification of MEPH and five of its Phase I metabolites was validated in human fingerprint sweat.

2.6.7.1 Selectivity

No interferences were observed in the extracted blank matrix.

2.6.7.2 Linearity

Mean linearity of $r^2 > 0.996$ was achieved for all analytes in all three validation runs.

2.6.7.3 LOD and LOQ

LOD of 50 pg/mL (LOQ of 200 pg/mL) for MEPH, NOR, DHNM; LOD of 40 pg/mL (LOQ of 160 pg/mL) for DHM; LOD of 25 pg/mL (LOQ of 100 pg/mL) for HYDROXY, 4-CARBOXY were achieved in this assay. Table 2-37 shows calibration parameters for all analytes.

Table 2-37. LOD, LOQ, calibration range and calibration parameters for mephedrone and its metabolites in fingerprint sweat

Analyte	**LOD** (ng/mL)	**LOQ** (ng/mL)	**Range** (ng/mL)	**Intercept ± SD** (n=3)	**Slope ± SD** (n=3)	**r^2 ± SD** (n=3)
MEPH	0.05	0.2	0.2-50	0.021 ± 0.045	2.37 ± 0.15	0.998 ± 0.001
DHM	0.04	0.16	0.16-50	0.054 ± 0.007	2.83 ± 0.05	0.999 ± 0.001
NOR	0.05	0.2	0.2-50	-0.028 ± 0.014	1.43 ± 0.24	0.998 ± 0.002
HYDROXY	0.025	0.1	0.1-50	0.027 ± 0.058	2.51 ± 0.76	0.997 ± 0.002
4-CARBOXY	0.025	0.1	0.1-50	0.009 ± 0.073	3.36 ± 1.14	0.996 ± 0.001
DHNM	0.05	0.2	0.2-50	-0.028 ± 0.039	2.31 ± 0.09	0.999 ± 0.000

2.6.7.4 Precision and accuracy

Intra-day and inter-day precision and accuracy results, summarised in Table 2-38, were found to be within the acceptance criteria. The intra-day inaccuracy was within ± 15% of the target concentration while accuracy ranged from 96.6-115% for MEPH, 101-105% for DHM, 90.9-106% for NOR, 85.6-111% for HYDROXY, 85.5-108% for 4-CARBOXY and 88.3-107% for DHNM. The intra-day imprecision was ≤ 13.8% and ranged from 0.523-5.10% for MEPH, 1.58-4.48% for DHM, 2.04-8.95% for NOR, 2.64-9.09% for HYDROXY, 2.51-13.8% for 4-CARBOXY and 0.906-5.06% for DHNM. Inter-day precision and accuracy results were acceptable over the validated range with %CV ≤ 14.5% and inaccuracy within ± 7.50% of the target concentration.

*Table 2-38. Precision and accuracy at QC Low, QC Med and QC High for all analytes in fingerprint sweat; * average value of 18 measurements over 3 days*

Analyte	**True value** (ng/mL)	**Mean** (ng/mL), **%CV**, **% accuracy**			
		Day 1 n=6	**Day 2 n=6**	**Day 3 n=6**	**Inter-day n=18** *
MEPH	0.8	0.800 1.31% 100%	0.854 3.44% 107%	0.805 5.10% 101%	0.820 4.58% 102%
	10	10.4 2.76% 104%	10.5 3.89% 105%	9.92 2.28% 99.2%	10.2 3.78% 102%
	40	40.6 0.523% 101%	45.8 1.06% 115%	38.6 3.11% 96.6%	42.5 9.61% 106%
DHM	0.4	0.410 3.07% 103%	0.418 2.62% 104%	0.411 2.23% 103%	0.413 2.63% 103%
	5	5.03 1.79% 101%	5.10 2.20% 102%	5.05 2.43% 101%	5.06 2.10% 101%
	40	41.0 4.48% 103%	41.0 1.58% 102%	41.9 3.41% 105%	41.9 7.37% 105%
NOR	0.8	0.774	0.805	0.745	0.775

		5.12%	3.68%	8.95%	6.66%
		96.7%	101%	93.1%	96.8%
		9.13	9.51	9.09	9.25
	10	4.71%	5.28%	2.87%	4.74%
		91.3%	95.1%	90.9%	92.5%
		36.6	38.7	42.6	40.3
	40	2.04%	2.66%	5.25%	14.5%
		91.6%	96.6%	106%	101%
		0.490	0.519	0.491	0.500
	0.5	5.91%	2.64%	5.53%	3.21%
		98.1%	104%	98.2%	100%
		10.1	8.92	11.1	10.0
HYDROXY	10	3.14%	8.43%	4.15%	10.7%
		101%	89.2%	111%	100%
		41.6	42.9	34.2	39.6
	40	8.28%	3.64%	9.09%	11.8%
		104%	107%	85.6%	98.9%
		0.451	0.503	0.476	0.476
	0.5	10.7%	5.41%	6.91%	5.49%
		90.1%	101%	95.2%	95.3%
		9.58	8.63	10.6	9.60
4-CARBOXY	10	2.51%	8.05%	2.91%	10.1%
		95.8%	86.3%	106%	96.0%
		39.8	43.1	34.2	39.0
	40	13.4%	7.58%	13.8%	11.5%
		99.4%	108%	85.5%	97.6%
		0.742	0.832	0.797	0.790
	0.8	5.06%	1.99%	4.96%	1.12%
		92.7%	104%	99.6%	98.7%
		8.83	10.1	9.80	9.57
DHNM	10	3.67%	2.47%	1.83%	0.484%
		88.3%	101%	98.0%	95.7%
		38.3	42.7	42.3	41.7
	40	3.62%	0.906%	2.53%	1.44%
		95.8%	107%	106%	104%

2.6.7.5 Recovery and matrix effect

As shown in Table 2-39, recovery was found to be greater than 60.6 ± 6.16% for all analytes, with NOR showing the lowest recovery of 60.6 ± 6.16% and 63.5 ± 4.32% at QC Low and QC High, respectively. The best recovery of 91.3 ± 5.53% and 91.1 ± 7.57% at

QC Low and QC High, respectively, was achieved for DHM. IS-corrected matrix effect values were within ± 7.3% at both QC levels for all analytes, showing no significant matrix effects affecting the assay.

Table 2-39. Analyte recovery and matrix effect at QC Low and QC High in fingerprint sweat

Analyte	Recovery (%CV), n=6		Matrix Effect (%CV), n=6	
	QC Low	QC High	QC Low	QC High
MEPH	72.2% (4.27%)	61.9% (5.67%)	101% (1.02%)	96.6% (1.77%)
DHM	91.3% (5.53%)	91.1% (7.57%)	101% (1.34%)	99.1% (0.375%)
NOR	60.6% (6.16%)	63.5% (4.32%)	99.8% (2.99%)	92.7% (1.43%)
HYDROXY	82.5% (5.60%)	62.7% (4.32%)	97.9% (5.02%)	96.1% (6.63%)
4-CARBOXY	83.4% (5.33%)	75.2% (4.24%)	98.6% (8.44%)	99.4% (6.24%)
DHNM	84.9% (8.11%)	70.6% (5.30%)	102% (3.63%)	93.6% (2.39%)

2.6.7.6 Carryover

Carryover was not observed.

2.6.7.7 Dilution integrity

Dilution integrity was only assessed for mephedrone. Due to the nature of fingerprint sweat collection, dilution was impractical at the beginning of an extraction as it would normally be the case with blood or urine. Moreover, only one sample was collected at each timepoint during the administration study making repeated analysis impossible. Therefore, dilution was performed after sample reconstitution, when an appropriate volume of the reconstituted sample was diluted 1 in 100 in the reconstitution solvent. As a result, internal standard was also diluted 1 in 100. In order to still be able to detect the internal standard after 1 in 100 dilution, concentration of the internal standard used in this method was increased to 250 ng/mL.

Good precision (9.91%) and accuracy (92.9%) was achieved for mephedrone following 1 in 100 dilution (Table 2-40).

Table 2-40. Dilution integrity (1 in 100) for mephedrone in fingerprint sweat

Analyte	**1 in 100 Dilution** (n=6)			
	True value (ng/mL)	**Calculated mean** (ng/mL)	**%CV**	**% accuracy**
MEPH	1	0.929	9.91%	92.9%

2.6.8 Head hair

A method for detection and quantification of MEPH and five of its Phase I metabolites was validated in human head hair.

2.6.8.1 Selectivity

No interferences were observed in the extracted blank matrix collected from individuals with dyed and natural hair.

2.6.8.2 Linearity

Mean linearity of $r^2 > 0.992$ was achieved for all analytes in all three validation runs.

2.6.8.3 LOD and LOQ

LOD of 10 pg/mg (LOQ of 40 pg/mg) for DHM and DHNM; 2.5 pg/mg (LOQ of 10 pg/mg) for HYDROXY; 5 pg/mg (LOQ of 20 pg/mg) for MEPH; and 1 pg/mg (LOQ of 4 pg/mg) for NOR and 4-CARBOXY were achieved in this assay. Table 2-41 shows calibration parameters for all analytes.

Table 2-41. LOD, LOQ, calibration range and calibration parameters for mephedrone and its metabolites in human head hair

Analyte	LOD (pg/mg)	LOQ (pg/mg)	Range (pg/mg)	Intercept ± SD (n=3)	Slope ± SD (n=3)	r^2 ± SD (n=3)
MEPH	5	20	20-200	0.595 ± 0.173	0.056 ± 0.043	0.997 ± 0.002
DHM	10	40	40-400	0.054 ± 0.016	0.013 ± 0.006	0.998 ± 0.002
NOR	1	4	4-400	0.244 ± 0.088	0.005 ± 0.001	0.998 ± 0.002
HYDROXY	2.5	10	10-400	0.356 ± 0.203	0.057± 0.074	0.992 ± 0.005
4-CARBOXY	1	4	4-400	0.076 ± 0.039	0.008 ± 0.005	0.995 ± 0.001
DHNM	10	40	40-200	0.725 ± 0.159	0.023 ± 0.004	0.998 ± 0.001

2.6.8.4 Precision and accuracy

Intra-day and inter-day precision and accuracy results, summarised in Table 2-42, were found to be within the acceptance criteria. The intra-day inaccuracy was within ± 15% of the target concentration while accuracy ranged from 85.8-111% for MEPH, 91.1-106% for DHM, 96.1-109% for NOR, 88.8-113% for HYDROXY, 87.1-101% for 4-CARBOXY and 87.8-104% for DHNM. The intra-day imprecision was ≤ 13.5% and ranged from 2.15-7.34% for MEPH, 2.30-12.5% for DHM, 2.57-8.77% for NOR, 4.43-12.2% for HYDROXY, 2.87-13.5% for 4-CARBOXY and 3.02-11.7% for DHNM. Inter-day precision and accuracy results were acceptable over the validated range with %CV ≤ 11.0% and inaccuracy within ± 7.70% of the target concentration.

*Table 2-42. Precision and accuracy at QC Low, QC Med and QC High for all analytes in head hair; * average value of 18 measurements over 3 days*

Analyte	True value (pg/mg)	Mean (pg/mg), %CV, % accuracy			
		Day 1 n=6	Day 2 n=6	Day 3 n=6	Inter-day n=18 *
MEPH	50	50.0 6.57% 99.9%	54.2 7.33% 108%	48.6 7.34% 97.2%	50.9 8.22% 102%
	100	100 2.59% 100%	95.0 4.58% 95.0%	85.8 2.15% 85.8%	93.7 7.30% 93.7%
	170	189 3.37% 111%	164 4.07% 96.2%	163 5.15% 96.0%	172 8.31% 101%
DHM	80	76.6 2.30% 95.8%	81.6 3.08% 102%	85.0 6.77% 106%	80.9 6.00% 101%
	190	182 3.60% 95.8%	195 5.59% 103%	175 5.85% 92.2%	185 6.48% 97.5%
	340	314 3.11% 92.4%	361 12.5% 106%	310 5.65% 91.1%	328 11.0% 96.6%
NOR	8	838 2.57% 105%	869 5.30% 109%	805 7.75% 101%	837 6.12% 105%
	40	38.5 3.35% 96.1%	40.7 4.78% 102%	42.8 6.59% 107%	40.7 6.65% 102%
	330	323 3.68% 97.8%	345 8.77% 104%	348 5.42% 105%	339 6.91% 103%
HYDROXY	20	20.1 8.01% 100%	20.3 12.0% 101%	22.6 6.99% 113%	20.9 10.2% 104%
	60	61.3 7.46% 102%	53.3 12.2% 88.8%	54.3 4.43% 90.4%	56.3 10.3% 93.8%
	280	278 7.45% 99.1%	260 9.71% 92.9%	267 5.24% 95.4%	269 7.43% 96.2%
4-CARBOXY	80	76.6 6.48% 95.8%	80.8 9.67% 101%	76.7 2.87% 95.9%	78.1 7.10% 97.6%

	190	190 4.93% 99.8%	180 5.72% 94.8%	188 10.4% 98.8%	186 7.38% 98.0%
	340	324 7.46% 95.3%	296 13.5% 87.1%	315 6.66% 92.7%	314 8.95% 92.3%
DHNM	5	493 7.28% 98.5%	496 3.42% 99.1%	501 3.50% 100%	497 4.95% 99.3%
	20	19.2 3.02% 96.1%	19.7 5.27% 98.6%	17.6 3.14% 87.8%	18.8 6.32% 94.2%
	160	155 4.73% 96.8%	166 11.7% 104%	157 6.37% 98.0%	159 8.52% 99.6%

2.6.8.5 Recovery and matrix effect

As shown in Table 2-43, recovery was found to be greater than 37.6 ± 3.73% for all analytes, except 4-CARBOXY for which recovery was 4.55 ± 1.82% at QC Low and 5.58 ± 8.90% at QC High. However, it needs to be noted that presented recovery is not a true recovery because the extent of spiked analytes being incorporated into hair is unknown.

IS-corrected matrix effect values were within ± 15% for all analytes at both QC levels, except for HYDROXY and 4-CARBOXY. HYDROXY was suppressed by 57.8 ± 20.5% at QC Low and by 22.8 ± 18.8% at QC High whereas 4-CARBOXY was suppressed by 31.6 ± 3.59% at QC Low and by 20.4 ± 4.93% at QC High (Table 2-43). In addition, imprecision of 20.5% at QC Low and 18.8% at QC High was observed for HYDROXY. This may be due to the lack of matching deuterated IS which is currently not commercially available. However, assay precision and accuracy for all analytes were within the acceptance criteria (Table 2-42).

Table 2-43. Analyte recovery and matrix effect at QC Low and QC High in human head hair

Analyte	Recovery (%CV), n=6		Matrix Effect (%CV), n=6	
	QC Low	QC High	QC Low	QC High
MEPH	51.8% (2.51%)	60.5% (6.43%)	115% (9.90%)	107% (8.49%)
DHM	66.9% (4.06%)	72.5% (4.07%)	114% (8.63%)	100% (6.50%)
NOR	37.6% (3.73%)	37.8% (3.45%)	101% (11.6%)	101% (13.1%)
HYDROXY	41.6% (3.78%)	39.5% (4.96%)	42.2% (20.5%)	77.2% (18.8%)
4-CARBOXY	4.55% (1.82%)	5.58% (8.90%)	68.4% (3.59%)	79.6% (4.93%)
DHNM	73.1% (3.18%)	71.5% (3.99%)	89.3% (11.8%)	102% (3.67%)

2.6.8.6 Carryover

Carryover was not observed.

2.6.8.7 Dilution integrity

Dilution integrity was not assessed.

CHAPTER 3

DETECTION AND PHARMACOKINETIC ANALYSIS OF MEPHEDRONE AND ITS METABOLITES IN WHOLE BLOOD, PLASMA AND DRIED BLOOD SPOTS

3.1 Whole blood (achiral method)

3.1.1 Detection of mephedrone and its metabolites in whole blood

Whole blood can provide information about recent drug use. Drugs are typically detected for up to 3 days in whole blood, but the detection window is dependent on the drug, route of administration and the dose. Even though whole blood is one of the most commonly tested biological matrices in forensic and clinical toxicology, there are no published studies on pharmacokinetics of mephedrone and/or its metabolites in this matrix following a controlled administration.

3.1.2 Whole blood aims

The aim of this study was to investigate for the first-time pharmacokinetics of mephedrone and its metabolites in human whole blood.

3.1.3 Experimental

3.1.3.1 Reagents

Mephedrone hydrochloride (MEPH), dihydro-mephedrone hydrochloride (DHM), mephedrone-d_3 hydrochloride (MEPH-d_3), dihydro-mephedrone-d_3 hydrochloride (DHM-d_3), 4-(2-aminoethyl) benzoic acid hydrochloride (AEBA) and sodium borohydride were purchased from Sigma-Aldrich (Dorset, UK). Nor-mephedrone hydrochloride (NOR) and mephedrone hydrochloride in powder form used for the human administration were purchased from Chiron (Trondheim, Norway). Hydroxytolyl-mephedrone hydrochloride (HYDROXY), 4-carboxy-mephedrone hydrochloride (4-CARBOXY) as well as nor-mephedrone hydrochloride used for the in-house synthesis of dihydro-nor-mephedrone (DHNM) were purchased from LGC Standards (Bury, UK). MEPH, MEPH-d_3, DHM, DHM-d_3 were purchased as certified reference materials. All reference standards were analysed in-house to verify their chemical structure.

All solvents were HPLC grade unless stated otherwise. Methanol (MeOH), isopropyl alcohol (IPA), dichloromethane (DCM), acetonitrile (LC-MS grade for the preparation of the mobile phase and HPLC grade for other uses), formic acid, acetic acid, sodium phosphate monobase, sodium phosphate diabase and ammonium hydroxide (0.88, 35%) were purchased from Fisher Scientific (Loughborough, UK). Ultrapure water (18 MΩcm) was prepared on an ELGA Purelab Maxima HPLC water purification system (High Wycombe, UK). Xtrackt® DAU High Flow (150 mg, 3 mL) cartridges were purchased from Chromatography Direct (Runcorn, UK).

3.1.3.2 Blank matrix collection

Drug-free whole blood was collected by trained phlebotomists into 5 mL vacutainers containing 12.5 mg of sodium fluoride and 10 mg of potassium oxalate (NaF/KOx). Ethical approval for the collection of drug-free matrix was granted by the Research

Ethics Committee at King's College London (HR 16/17 4237) and can be found in Appendix B.

3.1.3.3 Volunteer administration study and sample collection

Six healthy male volunteers (referred to here as M1-M6) nasally insufflated 100 mg of mephedrone hydrochloride supplied as a racemic mixture (purity: 96.3 ± 0.5%). Whole blood (5 mL) was collected into vacutainers containing NaF/KOx preservative at -10 min (0 h, before administration), 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 60 min, 75 min, 90 min, 105 min, 2 h, 2.5 h, 3 h, 5 h, 6 h, Day 2 and Day 3. Whole blood samples were stored at +4°C and were extracted within 2 days of sample collection. Stability of mephedrone and five of its Phase I metabolites in human whole blood (NaF/KOx) stored at +4°C and -20°C has been published by our research group [125].

3.1.3.4 Working solutions

Working solutions used for the preparation of the calibration curve were made in MeOH:water (50:50 v/v) at 4, 8, 16, 20, 100, 160, 200 ng/mL for MEPH, DHM, NOR, DHNM; 4, 10, 20, 100, 200, 400, 500 ng/mL for HYDROXY; and 40, 100, 200, 500, 1000, 1600, 2000 ng/mL for 4-CARBOXY. Working solution used for the preparation of the quality control (QC) samples at low, medium and high level were made in MeOH:water (50:50 v/v) at 5, 20, 160 ng/mL for MEPH, DHM, NOR, DHNM; 5, 40, 400 ng/mL for HYDROXY; and 50, 400, 1600 ng/mL for 4-CARBOXY. Internal standard (IS) solution containing MEPH-d_3 and DHM-d_3 at 50 ng/mL and AEBA at 500 ng/mL was prepared in MeOH:water (50:50 v/v). AEBA was used as an IS for 4-CARBOXY because it helped minimised matrix effects.

3.1.3.5 Calibration standards and quality control samples

Matrix-matched calibration standards containing MEPH, DHM, NOR, DHNM at 0.2, 0.4, 0.8, 1, 5, 8, 10 ng/mL; HYDROXY at 0.2, 0.5, 1, 5, 10, 20, 25 ng/mL; and 4-CARBOXY at 2, 5, 10, 25, 50, 80, 100 ng/mL were prepared by the addition of an appropriate volume of the working solution to whole blood. QC Low (0.250 ng/mL for MEPH, DHM, NOR, DHNM, HYDROXY; and 2.5 ng/mL for 4-CARBOXY), QC Med (1 ng/mL for MEPH, DHM, NOR, DHNM; 2 ng/mL for HYDROXY; and 20 ng/mL for 4-CARBOXY) and QC High (8 ng/mL for MEPH, DHM, NOR, DHNM; 20 ng/mL for HYDROXY; and 80 ng/mL for 4-CARBOXY) were prepared by the addition of an appropriate volume of the working solution to whole blood.

Calibration standards and QCs were prepared fresh on the day of sample analysis. Blanks containing whole blood but no IS and one sample containing whole blood and IS were also prepared and taken through the extraction.

3.1.3.6 Sample preparation

One hundred microliters of whole blood (NaF/KOx) was extracted using solid phase extraction (SPE). Where dilution was required, samples were diluted 1 in 20 in the blank matrix alongside 3 additional QCs prepared in the same manner. Ten microliters of the IS was added to the samples, calibration standards and QCs. All samples were vortex mixed and 1 mL of 0.1 M phosphate buffer (pH 6.0) was added. After conditioning the Xtrackt® SPE cartridges (mixed mode cation exchange containing C8 and benzoylsulfonate anion) with 2 mL of MeOH and 2 mL of 0.1 M phosphate buffer (pH 6.0), samples were loaded and washed with 2 mL of 0.1 M acetic acid(aq) followed by 2 mL of MeOH. Samples were eluted with 4 x 1 mL of DCM:IPA:ammonium hydroxide (78:20:2 v/v/v) and dried under nitrogen at 50°C. Samples were reconstituted with 100 µL of 0.1% formic acid in ACN:water (10:90 v/v).

3.1.3.7 LC-MS/MS conditions

The analysis was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Waters Xevo TQ-S triple quadrupole mass spectrometer (Manchester, UK) coupled to Waters Acquity ultra performance liquid chromatograph system equipped with a CTC 2777 open architecture autosampler (Waters, UK).

Extracted samples were analysed using electrospray ionisation operated in positive ion mode. The source temperature was set to 150°C. The desolvation gas flow rate was 1000 L/h at a temperature of 500˚C, capillary voltage was set to 2.22 kV, cone voltage was 45 V and source offset was 84 V. The cone gas flow rate was set to 150 L/h, the nebuliser gas flow was 7.00 bar and the collision gas flow rate was 0.25 mL/min.

Mephedrone metabolites and deuterated internal standards were monitored using selected reaction monitoring (SRM) as detailed in Table 3-1. In order to maximise sensitivity, all analytes except for 4-CARBOXY and HYDROXY had their dehydration products chosen as target precursor ions due to significant in-source fragmentation which is commonly observed in synthetic cathinones [74,125].

Table 3-1. The retention time, SRM transitions and collision energy for each ion

** denotes a quantifying transition*

*** denotes dehydrated precursor ions*

Analyte	**Retention time** (min)	**Precursor ion** (m/z)	**Product ion** (m/z)	**Collision energy** (V)	**Internal standard**
MEPH	5.85	160.4 **	145.1*	15	MEPH-d_3
			144.1	33	
			91.1	28	
MEPH-d_3	5.85	163.4 **	148.4	19	
DHM	5.38	162.4 **	147.3*	19	DHM-d_3
			131.4	17	

			91.3	26	
DHM-d_3	5.38	165.4 **	150.3	18	
NOR	5.00	146.0 **	131.1	25	MEPH-d_3
			130.1*	25	
			119.0	15	
HYDROXY	1.98	194.1	158.1	17	DHM-d_3
			146.0*	17	
			131.1	23	
4-CARBOXY	2.06	208.0	146.0*	13	AEBA
			144.1	28	
			130.1	31	
DHNM	4.45	148.1 **	131.1*	13	MEPH-d_3
			116.2	23	
			91.1	25	
AEBA	1.77	166.1	149.1	10	

Chromatographic separation was performed on a 2.1 mm x 150 mm, 1.8 µm, pentafluorophenylpropyl (PFPP) Selectra® column (Bristol, US) held at 60°C. The strong needle wash was 0.3% formic acid in MeOH and the weak needle wash was 0.01% formic acid in acetonitrile:water (10:90 v/v). The flow rate was 0.5 mL/min with 0.3% formic acid in water as mobile phase A and 0.3% formic acid in acetonitrile as mobile phase B. The start of the gradient was at 85% mobile phase A. Mobile phase B was then increased to 55% over 11 min and was held for 2 min. Over the next 0.5 min the gradient returned to the starting condition and the column was re-equilibrated at 85% mobile phase A for the remaining 1.5 min. The total run time was 15 min. The injection volume was 20 µL and the data was acquired using MassLynx software (Version 4.1). TargetLynx (version 4.1) was used for data processing and quantification.

3.1.3.8 Pharmacokinetic calculations

Please refer to Section 2.3 in Chapter 2.

3.1.3.9 Validation procedure

Please refer to Section 2.5 in Chapter 2.

3.1.4 Results

3.1.4.1 Method validation

Please refer to Section 2.6.1 in Chapter 2.

3.1.4.2 Concentrations of mephedrone and its metabolites in whole blood (NaF/KOx)

Mean whole blood concentrations ± standard deviation (SD) for mephedrone and its metabolites in 6 participants are presented in Figure 3-1 - Figure 3-6. Individual plots and raw data can be found in Appendix E.

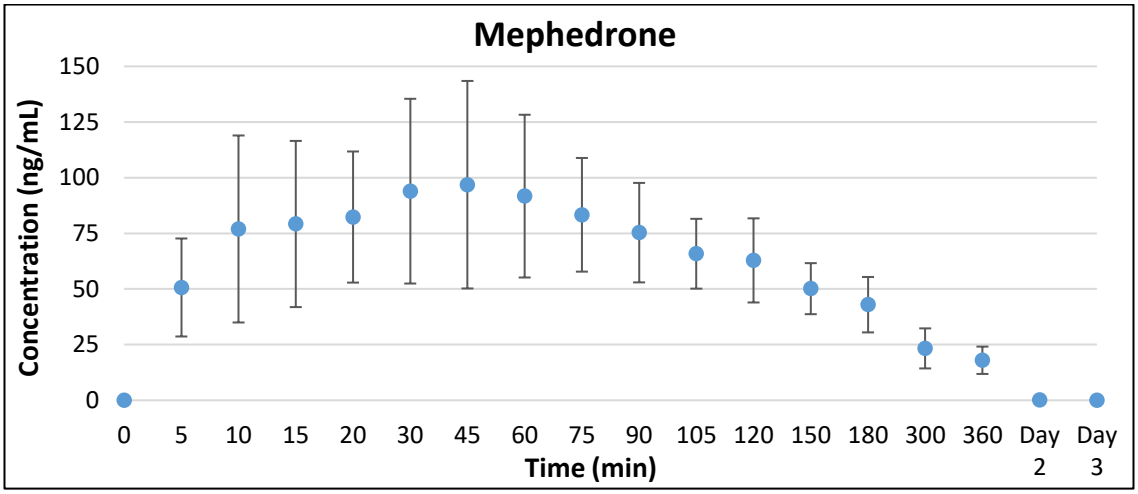


Figure 3-1. Mean mephedrone whole blood concentrations ± SD (n=6); note that mephedrone was detected in one participant at 0.212 ng/mL on Day 2

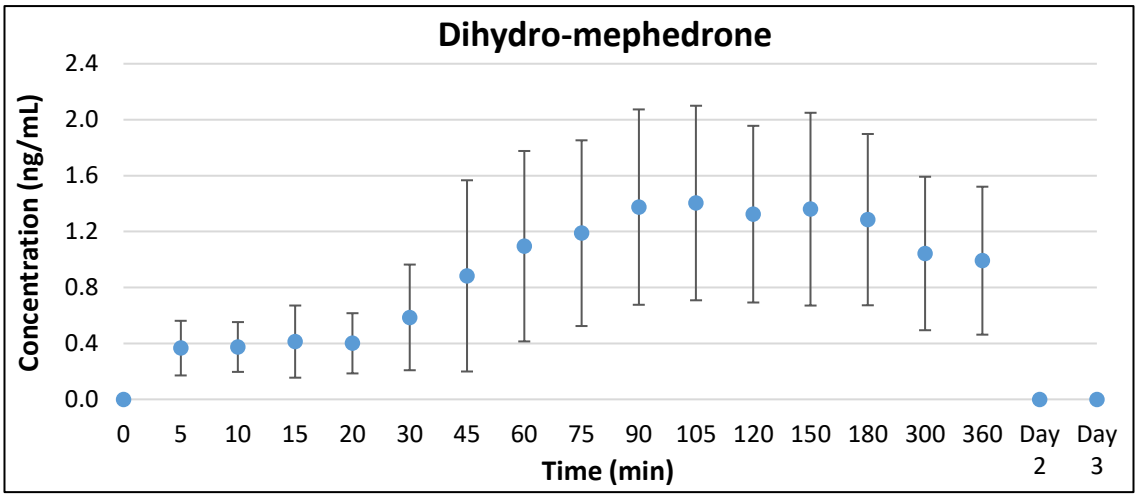


Figure 3-2. Mean dihydro-mephedrone whole blood concentrations ± SD (n=6)

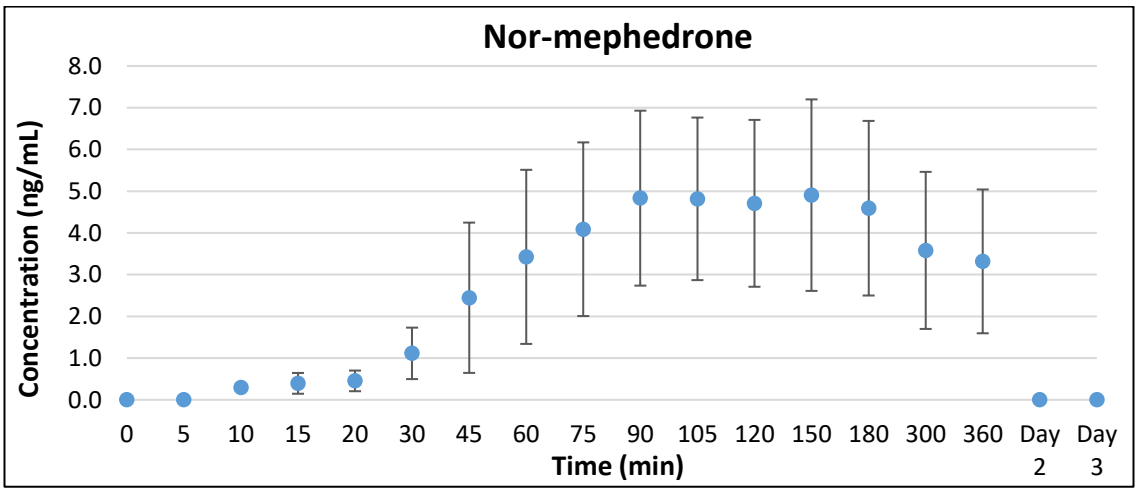


Figure 3-3. Mean nor-mephedrone whole blood concentrations ± SD (n=6); note that nor-mephedrone was detected in one participant at 10 min at 0.293 ng/mL

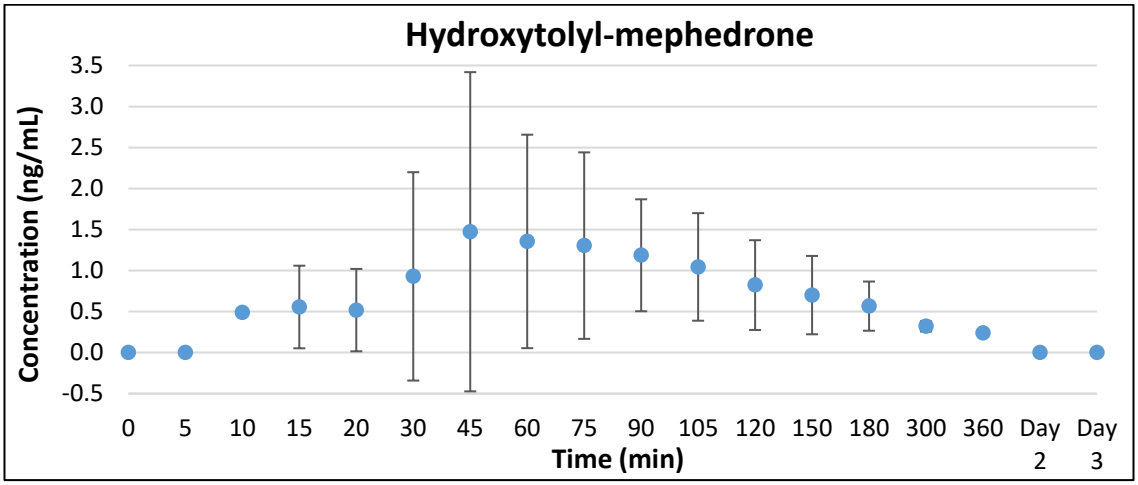


Figure 3-4. Mean hydroxytolyl-mephedrone whole blood concentrations ± SD (n=6); note that hydroxytolyl-mephedrone was detected in one participant at 10 min at 0.488 ng/mL

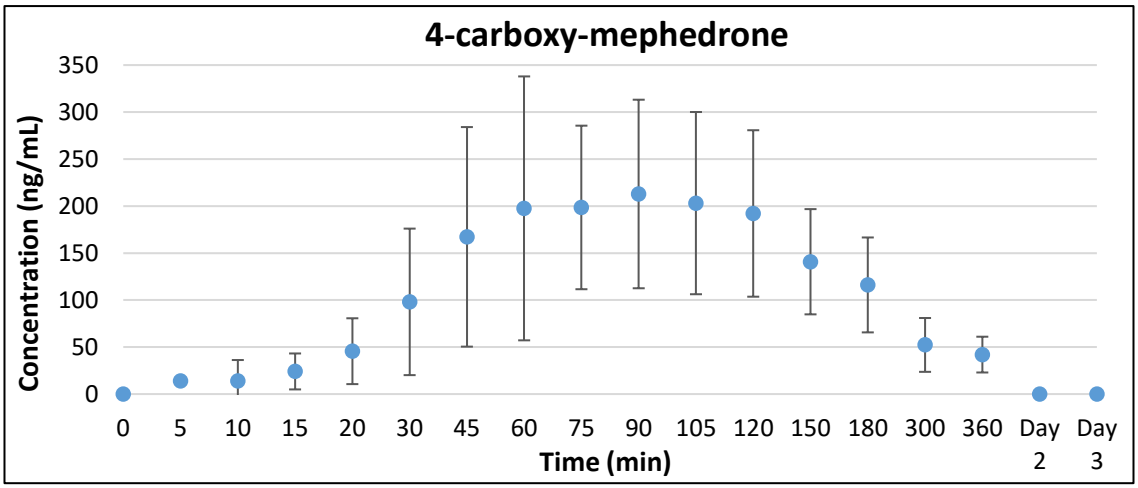


Figure 3-5. Mean 4-carboxy-mephedrone whole blood concentrations ± SD (n=6); note that 4-carboxy-mephedrone was detected in one participant at 5 min at 14.0 ng/mL

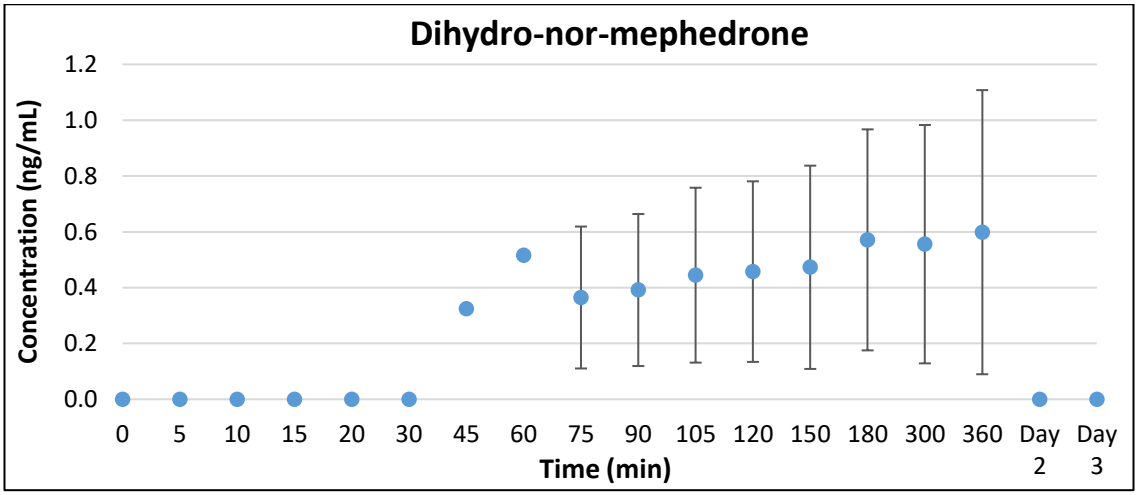


Figure 3-6. Mean dihydro-nor-mephedrone whole blood concentrations ± SD (n=6); note that dihydro-nor-mephedrone was detected in one participant at 45 min (0.324 ng/mL) and 60 min (0.516 ng/mL)

Following mephedrone administration, DHM and 4-CARBOXY were detectable in whole blood from 5 min, but 4-CARBOXY was only detected in one participant (M6) at this timepoint. NOR and HYDROXY were detected in whole blood after 10 min in M6 and after 20 min and 30 min in all other participants, respectively. DHNM appeared in whole blood after 45 min in M6, after 75 min in M1-M3 and after 150 min in M5. DHNM was not detected in M4. All analytes were present in whole blood until 360 min (6 h) with

mephedrone also being detectable just above the limit of quantification (LOQ) on Day 2 in M1.

3.1.4.3 Pharmacokinetic analysis

Whole blood drug concentrations were fitted with a single-dose, first-order elimination phase model and calculated mean pharmacokinetic (PK) parameters are summarised in Table 3-2. Individual PK data can be found in Appendix E.

Table 3-2. Mean ± SD pharmacokinetic data from the analysis of mephedrone and its metabolites in whole blood from 6 male participants; k_{el} and $t_{1/2}$ could not be calculated for DHNM because the elimination phase was not observed from the data

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)	CL (mL min^{-1} kg^{-1})	V (L kg^{-1})
MEPH	101 ± 45.4	55.0 ± 18.2	0.006 ± 0.001	2.12 ± 0.33	474 ± 150	54.1 ± 15.9	9.91 ± 2.96
DHM	1.45 ± 0.71	115 ± 33.8	0.002 ± 0.001	7.19 ± 4.19	16.0 ± 7.5	-	-
NOR	5.12 ± 2.16	133 ± 27.5	0.002 ± 0.001	6.09 ± 2.64	53.7 ± 24.0	-	-
HYDROXY	1.75 ± 1.81	65.0 ± 26.3	0.009 ± 0.006	1.52 ± 0.60	4.09 ± 3.89	-	-
4-CARBOXY	241 ± 113	85.0 ± 24.5	0.007 ± 0.001	1.70 ± 0.26	1104 ± 524	-	-
DHNM	0.607 ± 0.505	300 ± 73.5	-	-	8.04 ± 6.33	-	-

Mephedrone showed rapid absorption (T_{max} of 55.0 ± 18.2 min) and a relatively fast half-life ($t_{1/2}$) of 2.12 ± 0.33 h. Most mephedrone metabolites reached T_{max} shortly after the parent drug, except for NOR and DHNM which peaked later at 133 ± 27.5 min and

300 ± 73.5 min, respectively. 4-CARBOXY reached the highest concentration and had the largest area under the curve (AUC) of 241 ± 113 ng/mL and 1104 ± 524 ng mL^{-1} h, respectively. Another metabolite which was present in high abundance was NOR, reaching C_{max} of 5.12 ± 2.16 ng/mL and AUC of 53.7 ± 24.0 ng mL^{-1} h.

With regards to elimination, mephedrone and 4-CARBOXY showed similar kinetics, including similar $t_{1/2}$ (2.12 ± 0.33 h for mephedrone, 1.70 ± 0.26 h for 4-CARBOXY) and elimination rate constant (k_{el}; 0.006 ± 0.001 min^{-1} for mephedrone, 0.007 ± 0.001 min^{-1} for 4-CARBOXY). HYDROXY was eliminated with $t_{1/2}$ of 1.52 ± 0.60 h while DHM and NOR were eliminated with $t_{1/2}$ of 7.19 ± 4.19 h and 6.09 ± 2.64 h, respectively. $t_{1/2}$ and k_{el} were not determined for DHNM because the elimination phase was not observed from the data.

3.1.5 Discussion

Following mephedrone administration all analytes were successfully detected in whole blood, including NOR which is an active metabolite. NOR has been shown to cross the blood-brain barrier (BBB) in rats and to contribute to the psychoactive effects produced by mephedrone [91].

Mephedrone has been shown to be metabolised by cytochrome P450 2D6 (CYP2D6) which is mainly responsible for hydroxylation of the benzene ring (which leads to the formation of HYDROXY and then 4-CARBOXY) and N-demethylation of the secondary amine to form NOR. 4-CARBOXY and NOR were the most abundant metabolites in whole blood. 4-CARBOXY reached nearly 2.5 times higher AUC compared to mephedrone. NOR represented 11.3% of the AUC of mephedrone, followed by DHM (3.38%), DHNM (1.69%) and HYDROXY (0.84%). Pharmacokinetic values obtained from the analysis of whole blood samples cannot be compared with the literature because, to our knowledge, this is the first study that presents this data.

According to a review describing cases of clinical mephedrone intoxication, mean mephedrone whole blood concentration from fatal cases was 2,663 ng/mL (range: 51-22,000 ng/mL) compared to 166 ng/mL (range: 13-412 ng/mL) from non-fatal cases [407]. Additionally, in post-mortem blood mephedrone was found at 1200 ng/mL, 5700 ng/mL and 5500 ng/mL in the three cases where it was detected as the only substance [116,408]. In many of these cases the exact mephedrone dose was unknown and the route of administration varied between cases. In our study, mephedrone reached the C_{max} of 101 ± 45.4 ng/mL after a nasal insufflation. In other controlled human administration studies, where higher mephedrone doses (150 mg and 200 mg) were given orally, plasma (whole blood samples were not analysed) mephedrone concentration reached 123-159 ng/mL, which is in a similar range to the one associated with the acute non-fatal toxicities (mean: 166 ng/mL) [407].

3.1.6 Conclusion

For the first-time pharmacokinetics of mephedrone and its metabolites has been investigated in whole blood following a controlled drug administration. Mephedrone was rapidly absorbed, which may explain why mephedrone users tend to re-dose several times in a single session. All metabolites were detectable in whole blood for up to 6 h, with 4-CARBOXY and NOR being the most abundant.

3.2 Whole blood (chiral method)

3.2.1 Detection of mephedrone enantiomers in whole blood

The distribution of mephedrone enantiomers has not been investigated in human biological matrices following a controlled drug administration. The only available data comes from animal studies and the analysis of human urine collected from music festivals. In rats, R-mephedrone has been shown to result in more stimulant-like effects due to its predominant interaction with dopaminergic receptors [86]. A different study conducted by *Castrignanò et al.* investigated metabolism of mephedrone *in vitro* (in

human liver microsomes) and *in vivo* (in rat urine and human urine collected from music festivals) looking specifically at the enantiomeric ratio of R-mephedrone (R-MEPH) and S-mephedrone (S-MEPH) in the samples [88]. The researchers found human liver microsomes and human urine to be enriched with R-MEPH. In rat urine, S-MEPH was more abundant which likely highlights differences in metabolism between humans and rats. However, there is limited information about the purity of administered mephedrone to rats as authors only state that mephedrone powder was collected from the amnesty bins at a music festival in the UK in 2014 (at the time when mephedrone was sold as a racemic mixture).

3.2.2 Whole blood aims

The primary aim of the study was to carry out pharmacokinetic studies of S-MEPH and R-MEPH in human whole blood, and to investigate the possibility of enantioselective pharmacokinetics in humans. The secondary aim was to compare the data obtained for each enantiomer with total mephedrone concentrations found in whole blood (achiral method) in order to compare pharmacokinetic parameters between the analytes.

3.2.3 Experimental

3.2.3.1 Reagents

In addition to the reagents described in 3.1.3.1, diethylamine (DEA) and ethanol (EtOH; LiChrosolv grade) were purchased from Sigma-Aldrich (Dorset, UK). MeOH (Optima LC/MS) was purchased from Fisher Scientific (Loughborough, UK).

3.2.3.2 Blank matrix collection

Please refer to 3.1.3.2.

3.2.3.3 Volunteer administration study and sample collection

Whole blood samples from 5 participants (M2-M6) were used for chiral analysis. Samples from M1 were not analysed because the chiral method was not developed in time for that. Please refer to 3.1.3.3 for more details.

3.2.3.4 Working solutions

Working solutions used for the preparation of the calibration curve contained mephedrone only and were made in MeOH:water (50:50 v/v) at 160, 200, 400, 700, 1000, 2000, 4000 ng/mL. Working solution used for the preparation of the QC samples at low, medium and high level were also made in MeOH:water (50:50 v/v) at 200, 800, 3000 ng/mL. IS solution containing MEPH-d_3 at 50 ng/mL was prepared in the same solvent.

3.2.3.5 Calibration standards and quality control samples

Matrix-matched calibration standards containing mephedrone at 8, 10, 20, 35, 50, 100, 200 ng/mL were prepared by the addition of an appropriate volume of the working solution to whole blood. QC Low at 10 ng/mL, QC Med at 40 ng/mL and QC High at 150 ng/mL were also prepared by the addition of an appropriate volume of the working solution to whole blood.

Calibration standards and QCs were prepared fresh on the day of sample analysis. Blanks containing whole blood but no IS and one sample containing whole blood and IS were also prepared and taken through the extraction.

3.2.3.6 Sample preparation

Sample preparation described in 3.1.3.6 was followed, but samples were reconstituted in 0.1% DEA in EtOH:MeOH (20:80 v/v). Samples did not require dilution.

3.2.3.7 LC-MS/MS conditions

The same instrumentation and LC-MS/MS conditions were used as those described in 3.1.3.7, except for the LC method. Chromatographic separation was performed on a 150 mm x 3 mm, 3 µm CHIRALPAK AD-3 column (Illkirch, France) held at room temperature. The strong needle wash was 0.3% formic acid in MeOH and the weak needle wash was 0.01% formic acid in ACN:water (10:90 v/v). The flow rate was 0.1 mL/min with 0.1% DEA in EtOH:MeOH (20:80 v/v) as mobile phase used in an isocratic mode. The total run time was 10 min. Monitored SRM transitions are presented in Table 3-3.

Table 3-3. The retention time, SRM transitions and collision energy for S-MEPH and R-MEPH

** denotes a quantifying transition*

Analyte	**Retention time** (min)	**Precursor ion** (m/z)	**Product ion** (m/z)	**Collision energy** (V)	**Internal standard**
S-MEPH	5.9	160.4	145.1*	15	S-MEPH-d_3
			144.1	33	
			91.1	28	
R-MEPH	4.9	160.4	145.1*	15	R-MEPH-d_3
			144.1	33	
			91.1	28	
S-MEPH-d_3	5.9	163.4	148.4	19	
R-MEPH-d_3	4.9	163.4	148.4	19	

3.2.3.8 Determination of the enantiomeric ratio of R-mephedrone and S-mephedrone in mephedrone hydrochloride powder by circular dichroism

Ultraviolet and optical rotation spectra of mephedrone hydrochloride prepared at 4 mg/mL in MeOH were acquired on the Applied Photophysics Chirascan spectrometer (Leatherhead, UK). A Calcite polariser was mounted on the end of the photomultiplier tube detector inside the sample chamber for optical rotation measurement. A 10 mm Suprasil rectangular cell (Hellma UK Ltd, UK) was employed in the region of 600-250 nm. The instrument was flushed continuously with pure evaporated nitrogen throughout the experiment. The following parameters were employed for standard wavelength acquisition: 2 nm spectral bandwidth, 1 nm step size and 0.5 s instrument time per point. Single wavelength kinetic measurements were used: 2 nm spectral bandwidth, 200 s or 100 s time base with 100 points and accumulation of 5. Ten mg/mL of sucrose prepared in Milli-Q water was used to calibrate the instrument prior to samples measurements. All spectra were solvent baseline subtracted and measured at +20°C.

3.2.3.9 Determination of the enantiomeric ratio of R-mephedrone and S-mephedrone in mephedrone hydrochloride powder by LC-MS

Mephedrone solution prepared at 50 ng/mL in 0.1% DEA in EtOH:MeOH (20:80 v/v) was injected (n=3) on the chiral column as described in 3.2.3.7. Peak areas of the two separated enantiomers were compared.

3.2.3.10 Pharmacokinetic calculations

Please refer to Section 2.3 in Chapter 2.

3.2.3.11 Validation procedure

Please refer to Section 2.5 in Chapter 2.

3.2.4 Results

3.2.4.1 Method validation

Please refer to Section 2.6.2 in Chapter 2.

3.2.4.2 Enantiomeric ratio of R-mephedrone and S-mephedrone in mephedrone hydrochloride powder

The enantiomeric ratio of R-MEPH and S-MEPH in mephedrone hydrochloride powder administered to healthy volunteers in the study was determined by circular dichroism spectroscopy and LC-MS. The optical rotation produced a flat line in the region of 600-250 nm which showed that the mephedrone powder was supplied as a racemic mixture. This was further confirmed by the LC-MS analysis. Peak areas of R-MEPH and S-MEPH were 10404 ± 11 (n=3) and 10786 ± 23 (n=3), respectively, indicating an approximate 1:1 ratio. An example chromatogram showing the separation of the two enantiomers and their corresponding peak areas is presented in Figure 3-7. Peak areas of MEPH-d_3 also showed an approximate 1:1 ratio (data not shown here).

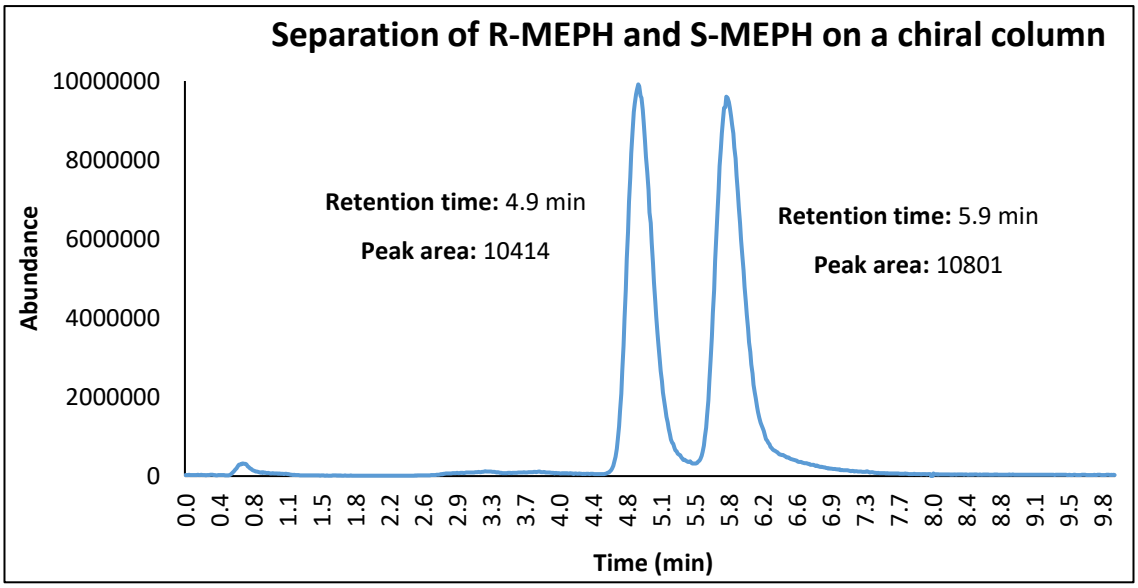


Figure 3-7. A chromatogram showing the separation of R-MEPH (first peak) and S-MEPH (second peak) on a chiral column

3.2.4.3 Concentration of S-mephedrone and R-mephedrone in whole blood (NaF/KOx)

In order to highlight individual changes in analyte concentration, Figure 3-8 - Figure 3-12 show concentration profiles for S-MEPH and R-MEPH in each participant (M2-M6). Individual raw data can be found in Appendix E.

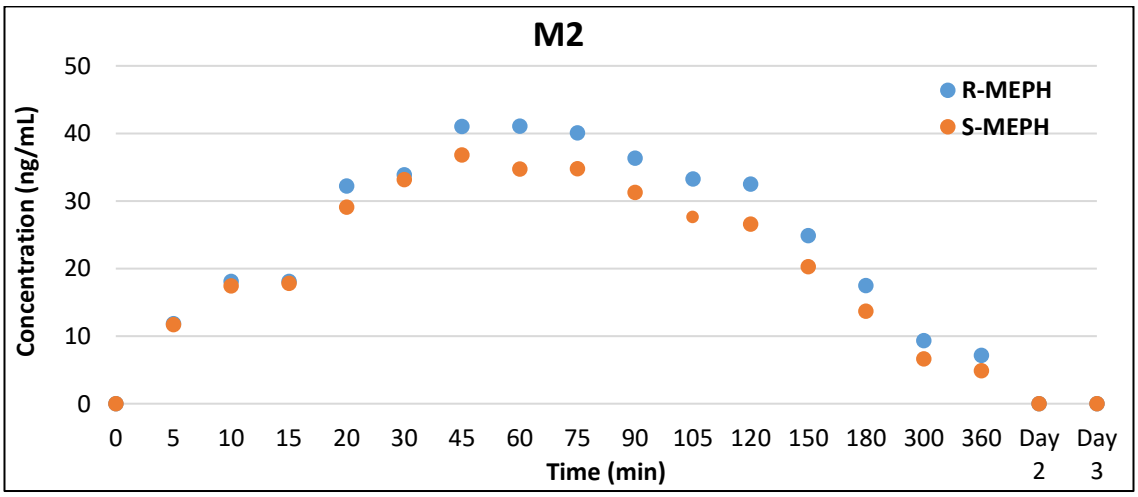


Figure 3-8. Concentrations of R-mephedrone and S-mephedrone in whole blood in M2

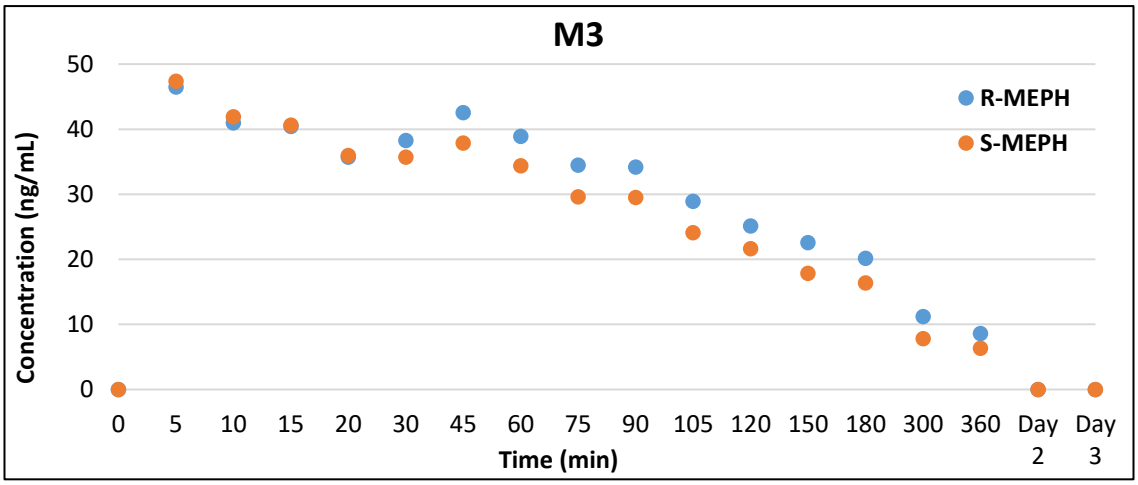


Figure 3-9. Concentrations of R-mephedrone and S-mephedrone in whole blood in M3

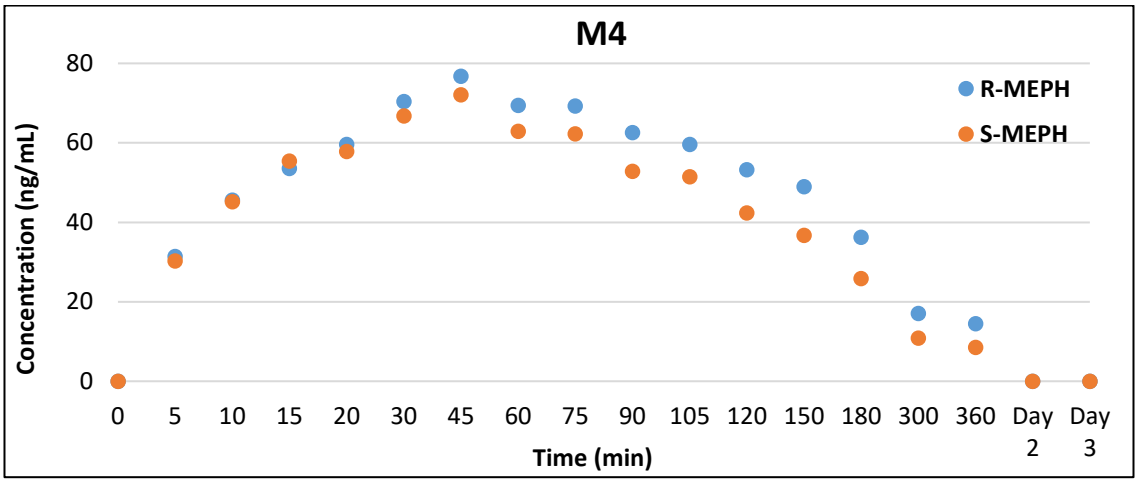


Figure 3-10. Concentrations of R-mephedrone and S-mephedrone in whole blood in M4

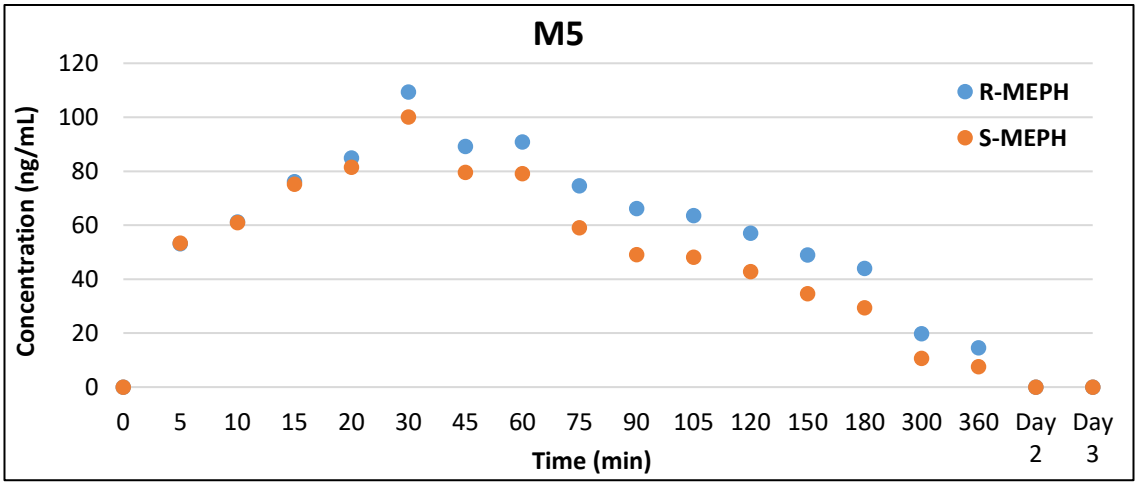


Figure 3-11. Concentrations of R-mephedrone and S-mephedrone in whole blood in M5

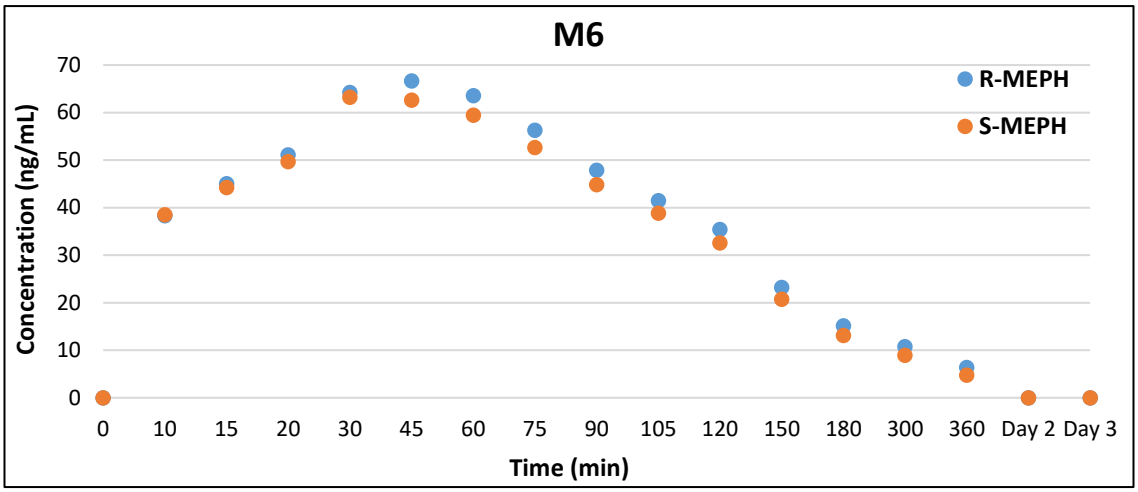


Figure 3-12. Concentrations of R-mephedrone and S-mephedrone in whole blood in M6

R-MEPH and S-MEPH were detected in whole blood between 5 min and 360 min, but R-MEPH reached higher concentrations between 20 min and 360 min. In M3, the first three samples collected at 5 min, 10 min and 15 min had high mephedrone concentrations which is likely due to contamination as mephedrone is not expected to reach C_{max} at 5 min. Elevated levels of mephedrone were also found in the 5 min, 10 min and 15 min samples from M3 in the achiral whole blood analysis, suggesting contamination during extraction or sample collection.

The enantiomeric fraction (EF) is used to show the proportion of enantiomers in a mixture. EF rather than the enantiomeric ratio is preferred for this determination [409,410] and is calculated according to Equation 3-1. EF was expected to be 0.5 if both enantiomers were present in whole blood samples at 1:1 ratio (i.e. racemic mixture).

Equation 3-1. Equation for calculating the enantiomeric fraction, where the enantiomer in the numerator is the (+) enantiomer

$$EF = \frac{peak\ area\ ratio\ of\ R\text{-}MEPH}{peak\ area\ ratio\ of\ R\text{-}MEPH\ and\ S\text{-}MEPH}$$

EF calculated for each participant at each timepoint between 5 min and 360 min is presented in Figure 3-13. EF values obtained from whole blood samples collected between 30 min and 360 min (where visible differences between the enantiomers were observed) ranged from 0.512 to 0.607 (mean: 0.535 ± 0.035). According to the one sample t-test (p-value < 0.0001 at 95% confidence interval), mean EF values were statistically different from the expected EF value of 0.5. Calibration standards and quality control samples had mean EF of 0.498 ± 0.014 across all runs.

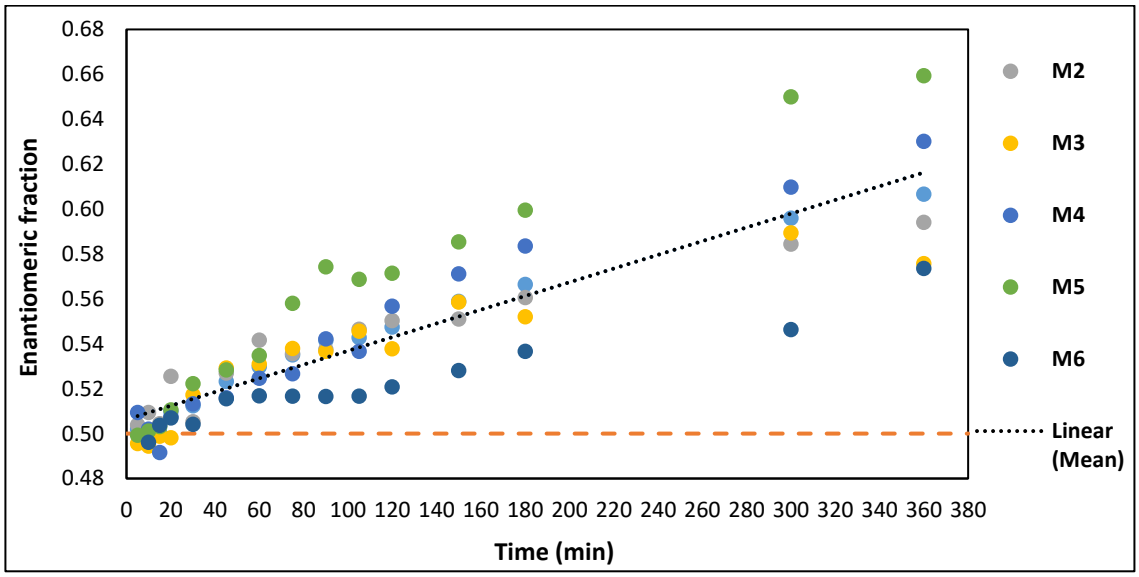


Figure 3-13. Changes in the EF with time post mephedrone administration for M2-M6; the orange dashed line at 0.500 shows the expected EF if both enantiomers were present in whole blood samples at 1:1 ratio; the black dotted line shows mean EF at each timepoint

3.2.4.4 Pharmacokinetic analysis

Whole blood drug concentrations were fitted with a single-dose, first-order elimination phase model and calculated mean pharmacokinetic parameters are summarised in Table 3-4 and Table 3-5.

Table 3-4. Mean ± SD pharmacokinetic data from the analysis of R-mephedrone in whole blood samples from 5 male participants

	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)	CL (mL min^{-1} kg^{-1})	V (L kg^{-1})
M2	41.1	60	0.006	1.92	192	114	18.9
M3	42.5	45	0.005	2.30	212	121	24.0
M4	38.0	45	0.006	2.00	179	157	27.2
M5	54.2	30	0.006	1.91	242	83.3	13.8
M6	66.7	45	0.007	1.56	209	144	19.5
Mean ± SD	48.5 ± 11.9	45.0 ± 10.6	0.006 ± 0.001	1.94 ± 0.26	207 ± 24	124 ± 29	20.7 ± 5.1

Table 3-5. Mean ± SD pharmacokinetic data from the analysis of S-mephedrone in whole blood sample from 5 male participants

	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)	CL (mL min^{-1} kg^{-1})	V (L kg^{-1})
M2	36.8	45	0.007	1.69	150	146	21.4
M3	37.9	45	0.006	2.01	171	150	26.1
M4	35.7	45	0.007	1.57	130	216	29.4
M5	49.7	30	0.008	1.45	157	128	16.2
M6	63.2	30	0.008	1.44	181	166	20.8
Mean ± SD	44.6 ± 11.8	39.0 ± 8.2	0.007 ± 0.001	1.63 ± 0.23	158 ± 20	161 ± 33	22.8 ± 5.1

Both enantiomers showed similar kinetics but S-MEPH peaked earlier at 39.0 ± 8.2 min and had shorter $t_{1/2}$ of 1.63 ± 0.23 h compared to 45.0 ± 10.6 min and 1.94 ± 0.26 h, respectively, for R-MEPH. Differences in the AUC and CL were also observed. R-MEPH resulted in 1.3 times greater AUC compared to S-MEPH and had lower clearance of 124 ± 29 mL min^{-1} kg^{-1} compared with 161 ± 33 mL min^{-1} kg^{-1} for the other enantiomer. Volume of distribution was similar for R-MEPH (20.7 ± 5.1 L kg^{-1}) and S-MEPH (22.8 ± 5.1 L kg^{-1}). Unpaired t-test was performed to check if the pharmacokinetic parameters

obtained for R-MEPH and S-MEPH were significantly different. At 95% confidence level, C_{max}, T_{max}, k_{el}, $t_{1/2}$, CL and V were not statistically different with the two-tailed p value of 0.6220, 0.3466, 0.0840, 0.0887, 0.0942 and 0.5345, respectively. However, the difference in the AUC between two enantiomers was deemed to be statistically significant (p value of 0.0075).

3.2.4.5 Comparison of the pharmacokinetic profile between total mephedrone and its enantiomers in whole blood

As expected, mean total mephedrone concentration in whole blood (achiral method) was roughly two times higher than the mean concentration of each individual enantiomer (Figure 3-14).

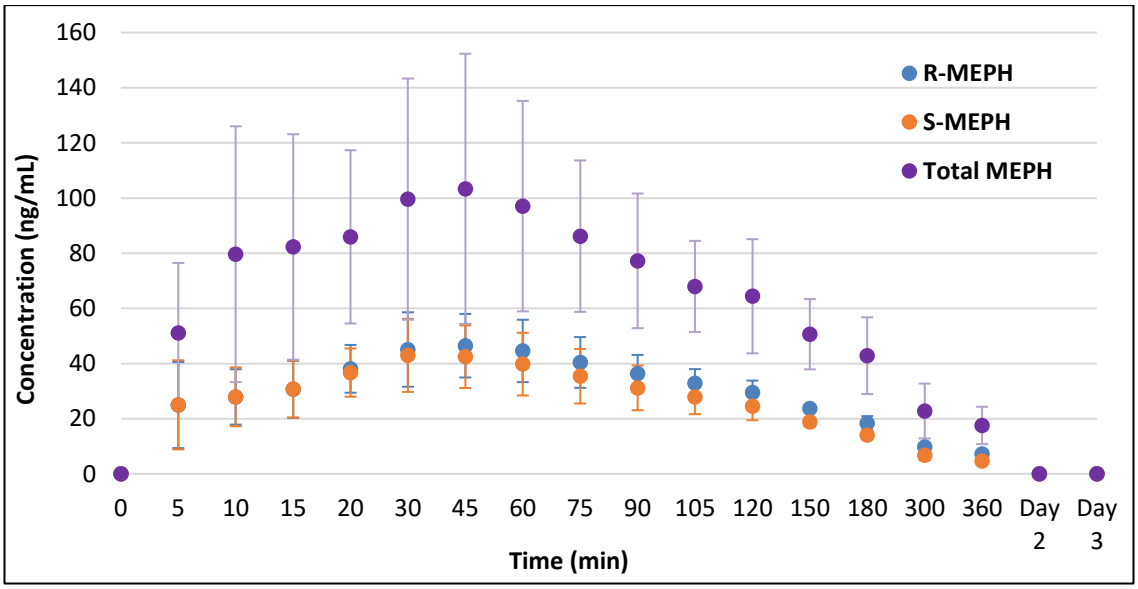


Figure 3-14. Mean concentration ± SD of R-mephedrone, S-mephedrone and total mephedrone in whole blood (M2-M5)

R-MEPH showed comparable pharmacokinetic parameters to those obtained for total mephedrone in whole blood (achiral method). Both peaked at approximately 50 min, had the same k_{el} of 0.006 ± 0.001 min^{-1} and nearly identical $t_{1/2}$ of 2.12 ± 0.33 h for total

mephedrone and 1.94 ± 0.26 h for R-MEPH. The C_{max} of 101 ± 45.4 ng/mL was observed for total mephedrone, which was 2.26 times higher than the C_{max} for S-MEPH and 2.08 times higher than the C_{max} for R-MEPH.

3.2.5 Discussion

Whole blood samples have been shown to be enriched with R-MEPH which reached higher C_{max}, T_{max} and had shorter $t_{1/2}$ than S-MEPH (the difference was not statistically significant). The AUC of R-MEPH was approximately 1.31 times greater than that of S-MEPH which was found to be statistically different. These results are in agreement with previous research that found R-MEPH to be a predominant analyte in pooled human urine samples and in *in vitro* experiments performed in human liver microsomes [87]. Moreover, changes in the EF over time presented in Figure 3-13 showed whole blood samples collected between 30 min and 360 min to be statistically different from the EF value of 0.5. Even though statistical difference was reported, it is not fully understood if it is likely to be clinically significant given the mean difference in EF and C_{max} of approximately 0.035 and 10%, respectively.

Differences in concentrations of S-MEPH and R-MEPH may be a result of pharmacokinetic processes occurring at different rates during drug absorption, distribution, metabolism or excretion [411]. Some drugs can be chemically or biochemically inverted *in vivo* in a unidirectional or bidirectional manner [412]. Moreover, polymorphic drug metabolism, gender, age, disease state and medications could all affect whole blood enantiomer concentrations, although given the design of this study polymorphic drug metabolism is likely to play the most significant role. CYP2D6, responsible for mephedrone metabolism, is subject to genetic polymorphism which causes variations in CYP2D6 enzymatic activity. People can be poor metabolisers (25% Caucasians), intermediate metabolisers, extensive metabolisers or ultrafast metabolisers (most commonly people of Middle Eastern descent) [413]. However, the mechanism leading to whole blood samples being enriched with R-MEPH in humans is not clear and warrants further studies.

3.2.6 Conclusion

This is the first-time enantiomers of mephedrone were separated on a CHRALPAK® AD-3 column and quantified in whole blood samples from a controlled drug administration. R-MEPH reached higher concentrations and had comparable pharmacokinetic parameters to total mephedrone. The enantiomeric fraction calculated from whole blood samples collected between 30 min and 360 min has been shown to be statistically different from the expected value of 0.5, suggesting enantioselective pharmacokinetics. However, it is not yet clearly understood if the difference is clinically significant.

3.3 Plasma

3.3.1 Detection of mephedrone and its metabolites in plasma

There has only been one dose-finding pilot study [75] and two controlled human mephedrone administration studies [96,97] published in the literature. The pilot study was a double-blind, randomised mephedrone administration study undertaken in Spain. The study recruited 9 occasional male users of psychostimulants who were given placebo, mephedrone or MDMA on 3 different occasions, with oral doses ranging from 50 mg to 200 mg. Mephedrone has also been administered twice before to human volunteers. *Papaseit et al.* conducted a double-blind, randomised, crossover, controlled oral administration study where 12 healthy male volunteers took 200 mg of mephedrone, 100 mg of MDMA or a placebo on 3 separate occasions [414]. More recently, six male healthy volunteers ingested 150 mg of mephedrone as part of a controlled administration study. Plasma samples were collected at 1 h, 2 h, 4 h, 6 h, and 8 h post drug administration and were analysed by GC-MS [97] and LC-MS [91]. In all studies mean mephedrone concentration peaked at approximately 1.25 h in plasma and displayed mean $t_{1/2}$ of 2.2 h. The study analysing samples on LC-MS also focused on the quantification of mephedrone metabolites. 4-CARBOXY reached the highest concentration after 1.2 ± 0.2 h followed by NOR (T_{max} = 1.5 ± 0.2 h) and DHM (T_{max} = 1.7

± 0.5 h) post drug administration. N-succinyl nor-mephedrone, being a derivative of NOR, peaked much later (T_{max} = 3.7 ± 0.3 h).

Depending on drug affinity to plasma proteins drugs might be free to distribute around the body or might be bound to proteins. The degree of protein-binding is an important consideration when drug concentrations are measured in different biological matrices. Whole blood is often the only specimen available in forensic investigations, such as driving under the influence of drugs and drug-facilitated crime, where immediate sample preparation does not usually take place [415]. In contrast, clinical laboratories routinely analyse plasma and serum samples. Therefore, it is important to compare obtained results between these two matrices with care as whole blood to plasma drug distribution ratios are not always known [416]. In particular little is known about whole blood to plasma distribution ratios of illicit drugs (except ethanol which has been extensively studied [417,418]). Consideration of this issue is especially relevant when comparing drug concentrations in whole blood to data from pharmacokinetic studies where serum or plasma has been analysed. To my knowledge, whole blood to plasma distribution ratios have not been reported before for mephedrone and its metabolites.

3.3.2 Plasma aims

The primary aim was to investigate pharmacokinetics of mephedrone and its metabolites in plasma (especially clearance and volume of distribution which have not been reported before) and to compare the data to that obtained from whole blood analysis. The secondary aim was to evaluate whole blood to plasma distribution ratios and to assess the correlation between analyte concentrations in these two matrices.

3.3.3 Experimental

3.3.3.1 Reagents

Please refer to 3.1.3.1.

3.3.3.2 Blank matrix collection

Please refer to 3.1.3.2 for more details. Drug-free, pooled human plasma (NaF/KOx) used for preparation of calibration standards, QCs and blanks was purchased from Sera Laboratories International Ltd (Burgess Hill, UK).

3.3.3.3 Volunteer administration study and sample collection

Please refer to 3.1.3.3 for more details. At each timepoint two aliquots of blood were collected, one of which was centrifuged at 2300 rpm for 10 min to harvest plasma.

3.3.3.4 Working solutions

Working solutions used for the preparation of the calibration curve were made in MeOH:water (50:50 v/v) at 10, 20, 25, 125, 250, 625, 1250 ng/mL for MEPH and 4-CARBOXY; 5, 12.5, 25, 125, 250, 625, 1250 ng/mL for DHNM; and 2.5, 10, 25, 125, 250, 625, 1250 ng/mL for HYDROXY, DHM and NOR. Working solution used for the preparation of the QC samples at low, medium and high level were made in MeOH:water (50:50 v/v) at 12.5, 125, 625 ng/mL for MEPH and 4-CARBOXY; 10, 125, 625 ng/mL for DHNM; and 5, 125, 625 ng/mL for HYDROXY, DHM and NOR. IS solution containing MEPH-d_3, DHM-d_3 at 50 ng/mL was prepared in MeOH:water (50:50 v/v).

3.3.3.5 Calibration standards and quality control samples

Matrix-matched calibration standards containing MEPH and 4-CARBOXY at 0.4, 0.8, 1, 5, 10, 25 and 50 ng/mL; DHNM at 0.2, 0.5, 1, 5, 10, 25 and 50 ng/mL; HYDROXY, DHM and NOR at 0.1, 0.4, 1, 5, 10, 25 and 50 ng/mL were prepared by the addition of an appropriate volume of the working solution to plasma. QC Low (0.5 ng/mL for MEPH and 4-CARBOXY; 0.4 ng/mL for DHNM and 0.2 ng/mL for HYDROXY, DHM and NOR), QC Med

(5 ng/mL for all analytes) and QC High (25 ng/mL for all analytes) were prepared by the addition of an appropriate volume of the working solution to plasma.

Calibration standards and QCs were prepared fresh on the day of sample analysis. Blanks containing plasma but no IS and one sample containing plasma and IS were also prepared and taken through the extraction.

3.3.3.6 Sample preparation

Sample preparation procedure described in 3.1.3.6 was followed but 250 µL of plasma was taken through the extraction.

3.3.3.7 LC-MS/MS conditions

DHM-d_3 was used as an IS for 4-CARBOXY. For other details please refer to 3.1.3.7.

3.3.3.8 Pharmacokinetic calculations

Please refer to Section 2.3 in Chapter 2.

3.3.3.9 Validation procedure

Please refer to Section 2.5 in Chapter 2.

3.3.4 Results

3.3.4.1 Method validation

Please refer to Section 2.6.3 in Chapter 2.

3.3.4.2 Concentrations of mephedrone and its metabolites in plasma (NaF/KOx)

Mean plasma concentrations ± SD for mephedrone and its metabolites in all 6 participants are presented in Figure 3-15 - Figure 3-20. Individual plots and raw data can be found in Appendix E.

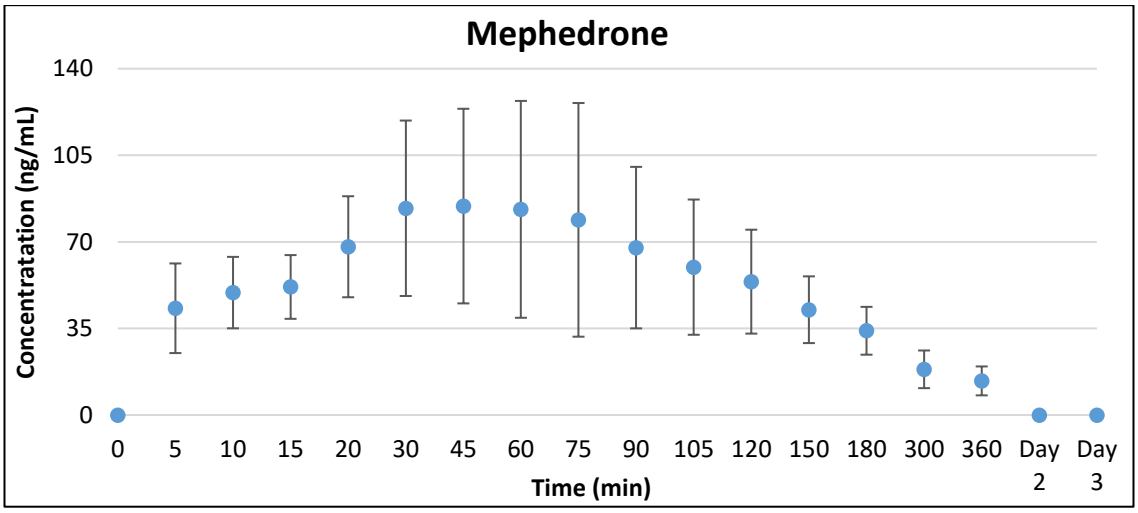


Figure 3-15. Mean mephedrone plasma concentrations ± SD (n=6)

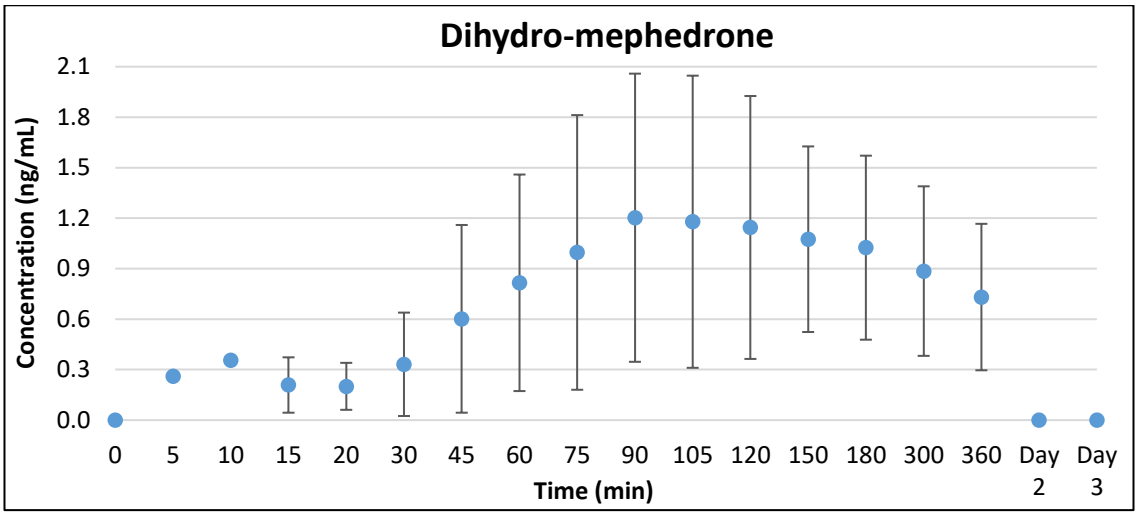


Figure 3-16. Mean dihydro-mephedrone plasma concentrations ± SD (n=6); note that dihydro-mephedrone was detected in one participant at 5 min (0.261 ng/mL) and 10 min (0.356 ng/mL)

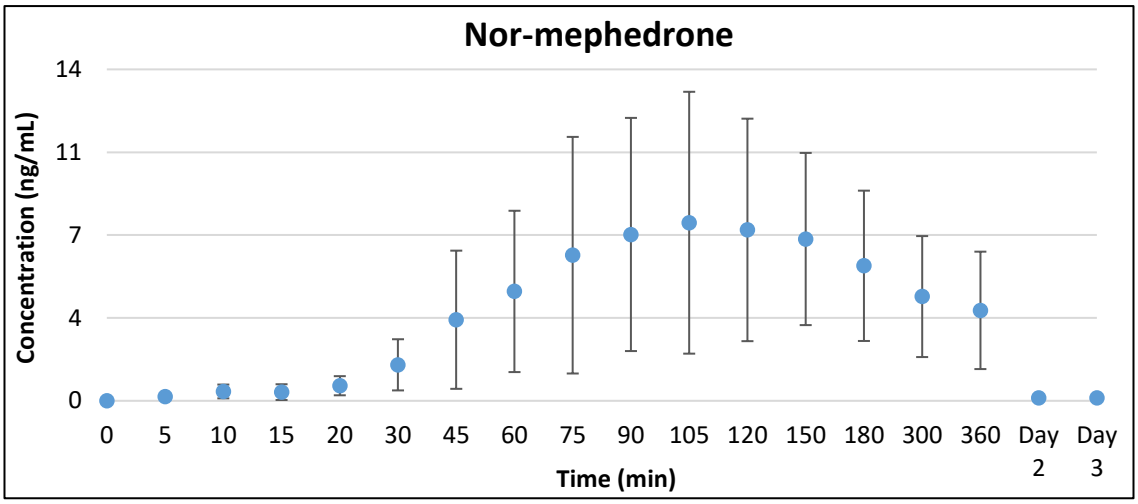


Figure 3-17. Mean nor-mephedrone plasma concentrations ± SD (n=6); note that nor-mephedrone was detected in one participant at 5 min (0.171 ng/mL) and in two participants on Day 2 (0.115 ng/mL and 0.138 ng/mL)

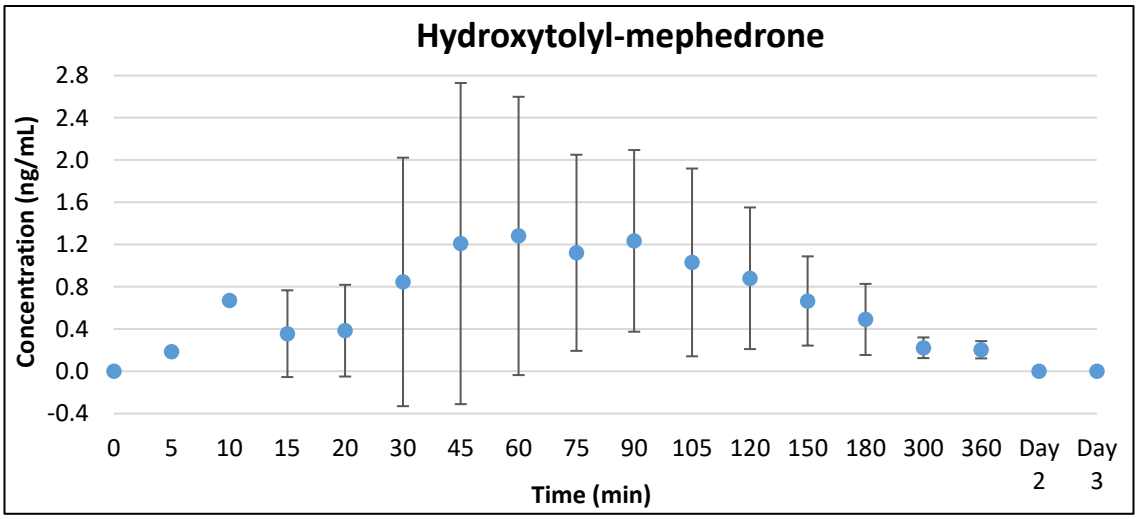


Figure 3-18. Mean hydroxytolyl-mephedrone plasma concentrations ± SD (n=6); note that hydroxytolyl-mephedrone was detected in one participant at 5 min (0.186 ng/mL) and 10 min (0.670 ng/mL)

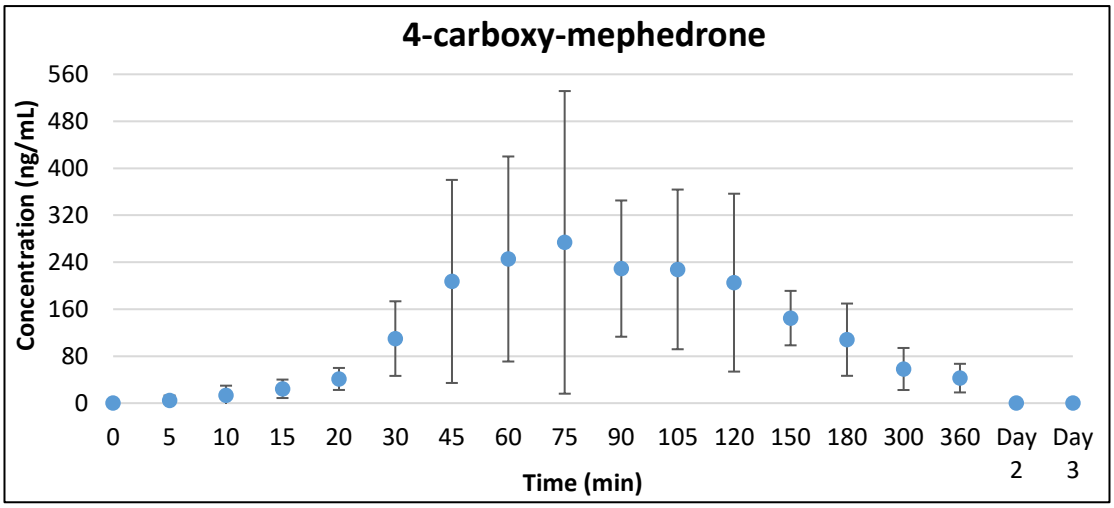


Figure 3-19. Mean 4-carboxy-mephedrone plasma concentrations ± SD (n=6)

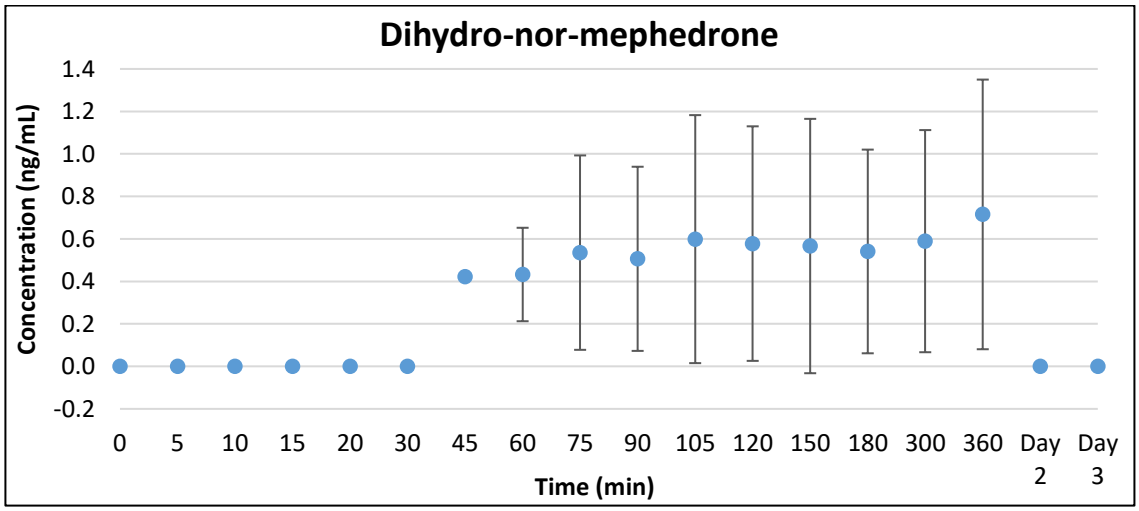


Figure 3-20. Mean dihydro-nor-mephedrone plasma concentrations ± SD (n=6); note that dihydro-nor-mephedrone was detected in one participant at 45 min (0.422 ng/mL)

Following the administration, MEPH and 4-CARBOXY were detectable in plasma from 5 min. DHM, NOR and HYDROXY were also present in plasma at 5 min but only in M6. In other participants DHM, NOR and HYDROXY were detectable after 30 min, 15 min and 20 min, respectively. DHNM appeared in plasma after 45 min in M6 and after 75 min in M1-M3. The analyte was not detected in M4 and M5. All analytes were present in plasma until 360 min with NOR also being detected on Day 2 in M1 and M3.

3.3.4.3 Whole blood to plasma distribution ratios

Whole blood to plasma distribution ratios for mephedrone and its metabolites were calculated from the C_{max} in both matrices (see Table 3-6).

Assuming the mean haematocrit value of 0.45, calculated median and mean were greater than 1 for mephedrone, DHM, HYDROXY and DHNM and smaller than 1 for NOR and 4-CARBOXY. %CV ranged from 8.5% for mephedrone to 33.6% for NOR.

Table 3-6. Mean, median, range, %CV and p-values obtained for the whole blood to plasma distribution ratios calculated from the C_{max}; p-value could not be calculated for DHNM because the sample size was too small (DHNM was not detected in M4 in either matrix and it was only detected in M5 in plasma)

Analyte	Mean	Median	Range	%CV	p-value
MEPH	1.14	1.11	1.02-1.27	8.5	0.018
DHM	1.26	1.30	0.885-1.54	18.8	0.043
NOR	0.750	0.765	0.433-1.13	33.6	0.045
HYDROXY	1.13	1.16	0.899-1.32	13.8	0.092
4-CARBOXY	0.899	0.973	0.571-1.06	21.1	0.248
DHNM	1.26	1.27	0.961-1.55	24.9	-

3.3.4.4 Correlation with whole blood

Correlation between analyte concentrations in whole blood and plasma in each participant is presented in Figure 3-21 - Figure 3-26 (the blue dotted line shows a trend line). Individual correlation can be found in Appendix E.

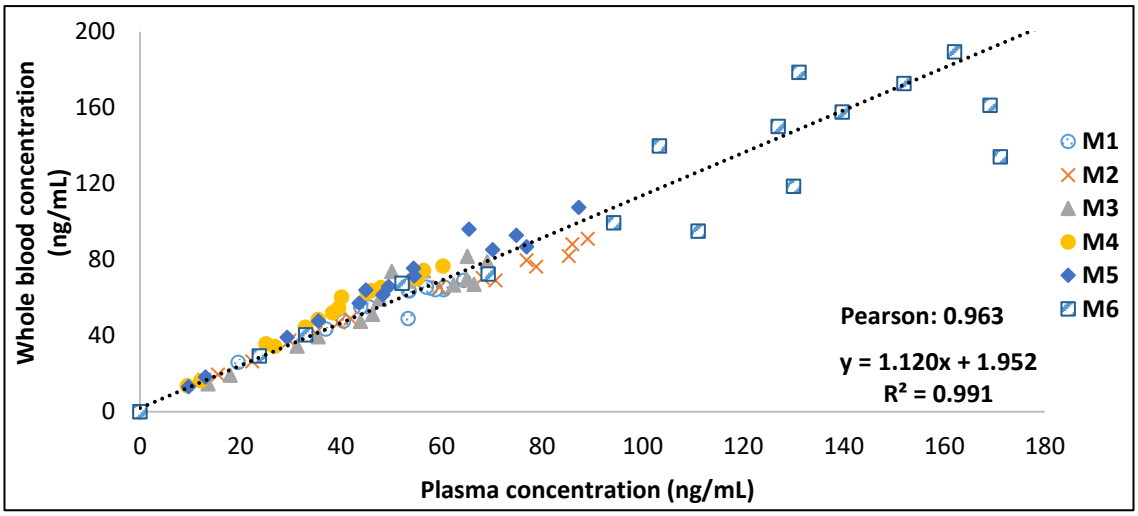


Figure 3-21. Correlation between plasma and whole blood concentrations for mephedrone

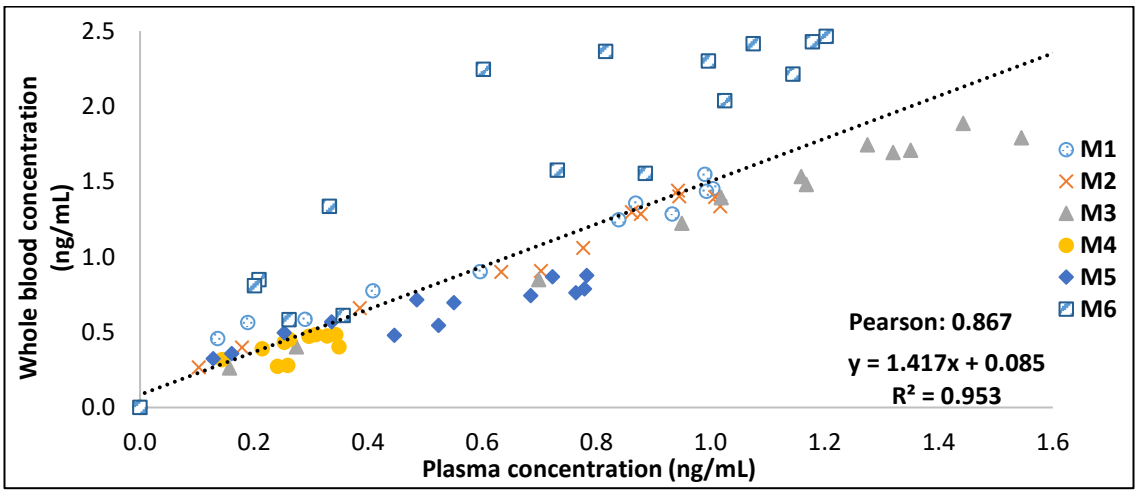


Figure 3-22. Correlation between plasma and whole blood concentrations for dihydro-mephedrone

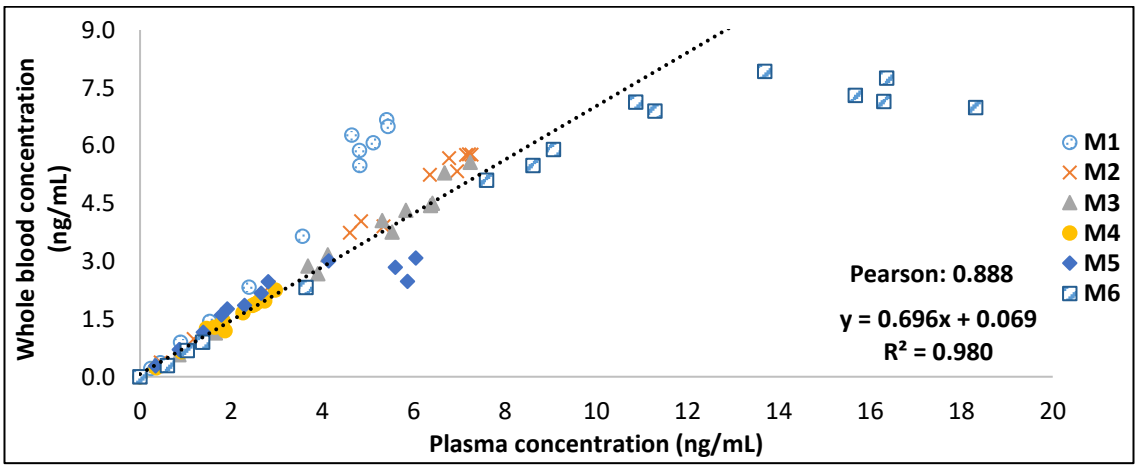


Figure 3-23. Correlation between plasma and whole blood concentrations for nor-mephedrone

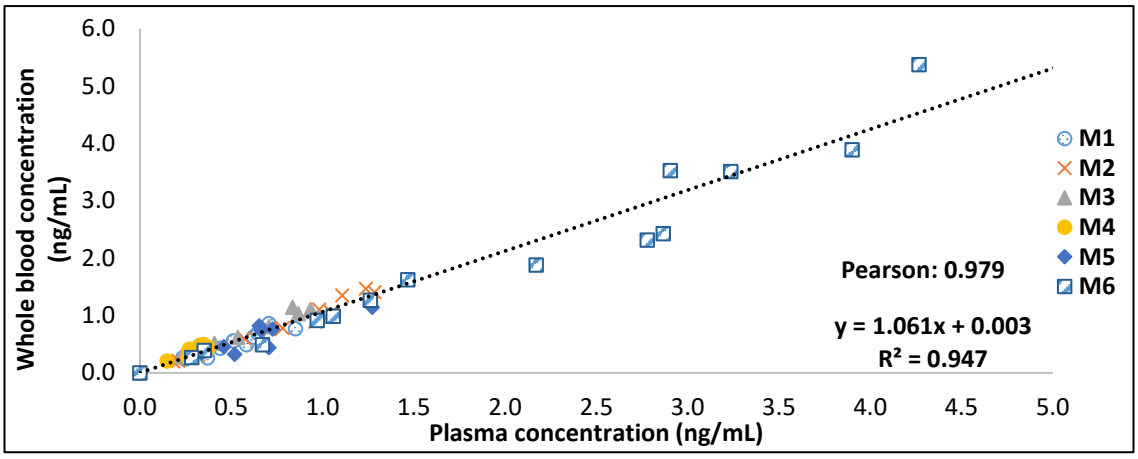


Figure 3-24. Correlation between plasma and whole blood concentrations for hydroxytolyl-mephedrone

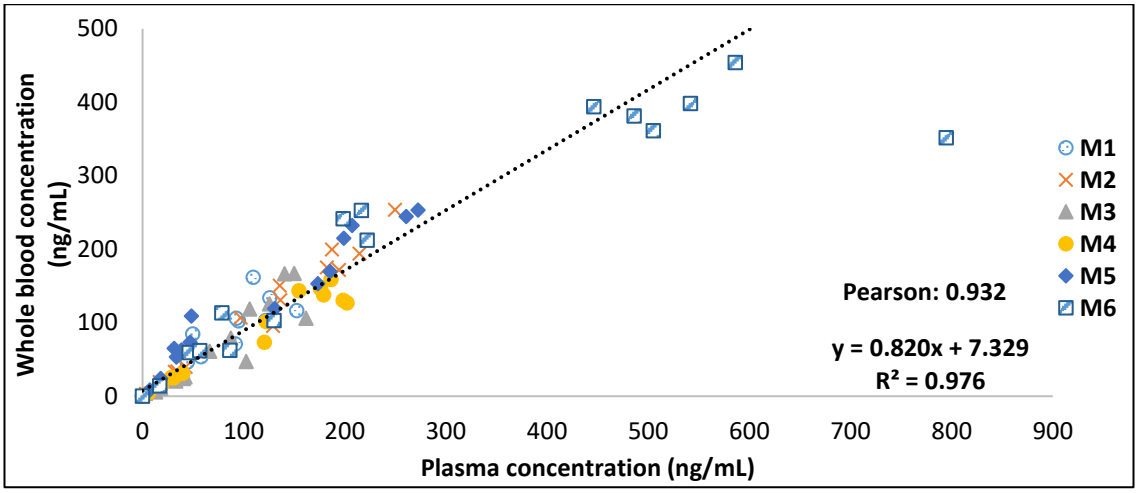


Figure 3-25. Correlation between plasma and whole blood concentrations for 4-carboxy-mephedrone

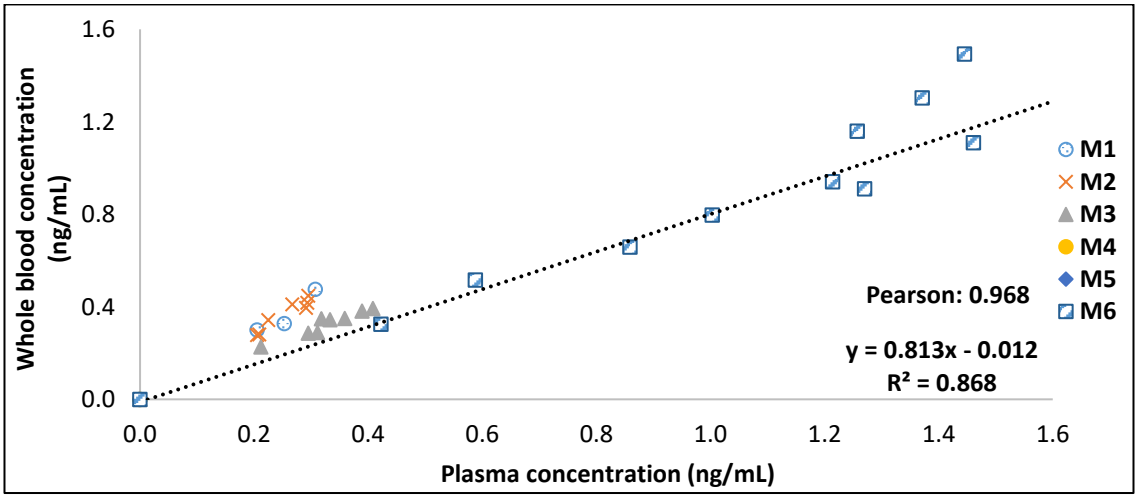


Figure 3-26. Correlation between plasma and whole blood concentrations for dihydro-nor-mephedrone

Concentrations of mephedrone and its metabolites appear to be significantly correlated between plasma and whole blood as evidenced by the Pearson correlation coefficient being greater than 0.867.

3.3.4.5 Whole blood and plasma - method comparison

Bland-Altman analysis was performed to examine the agreement between concentrations obtained in whole blood and plasma. Bland-Altman plots, presented in Figure 3-27 - Figure 3-32, show the difference between paired concentrations from plasma and whole blood samples plotted against the mean of the two concentrations calculated for each individual sample. Dotted lines represent the 95% limits of agreement (mean difference ± 2 SD).

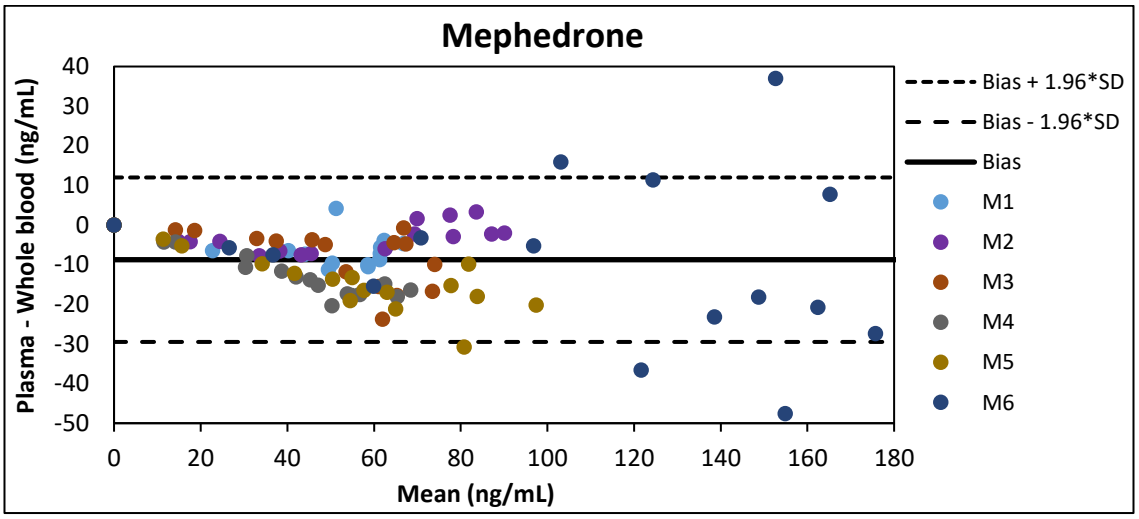


Figure 3-27. Bland-Altman analysis between plasma and whole blood for mephedrone

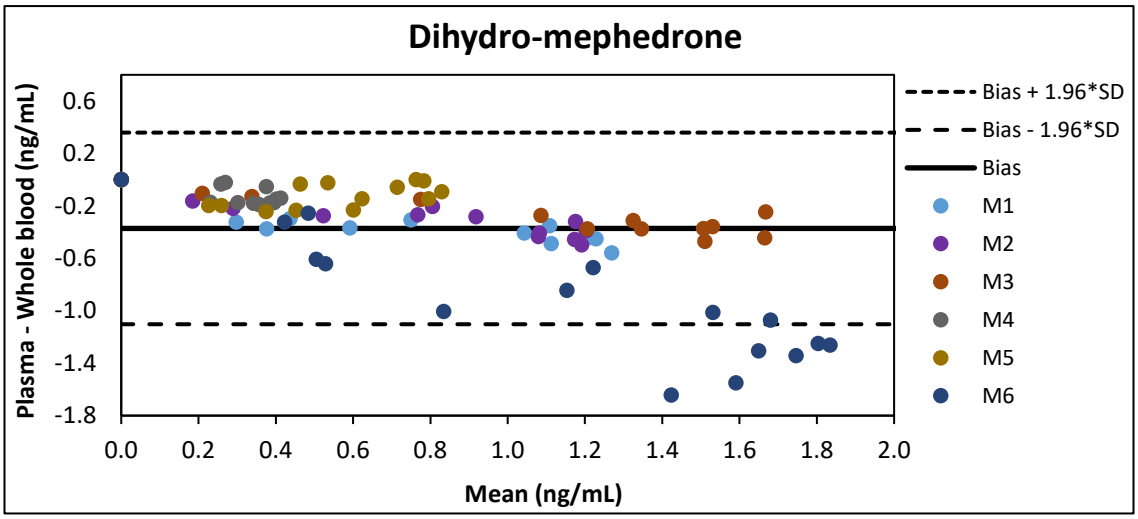


Figure 3-28. Bland-Altman analysis between plasma and whole blood for dihydro-mephedrone

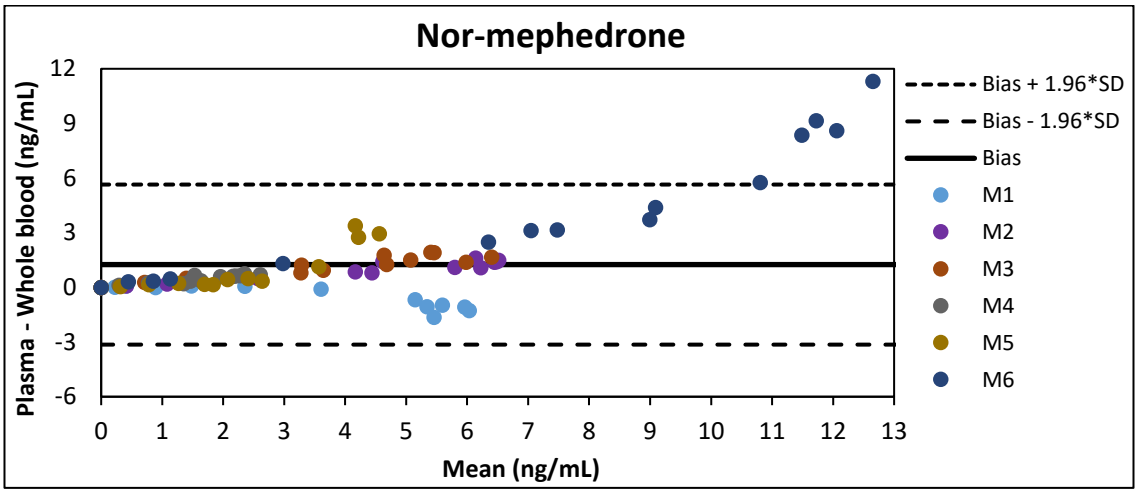


Figure 3-29. Bland-Altman analysis between plasma and whole blood for nor-mephedrone

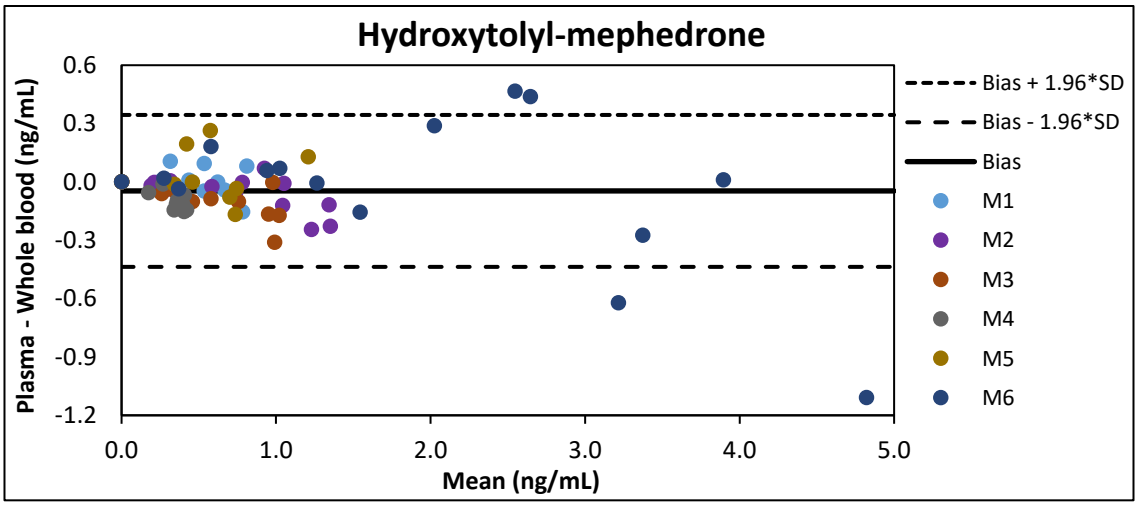


Figure 3-30. Bland-Altman analysis between plasma and whole blood for hydroxytolyl-mephedrone

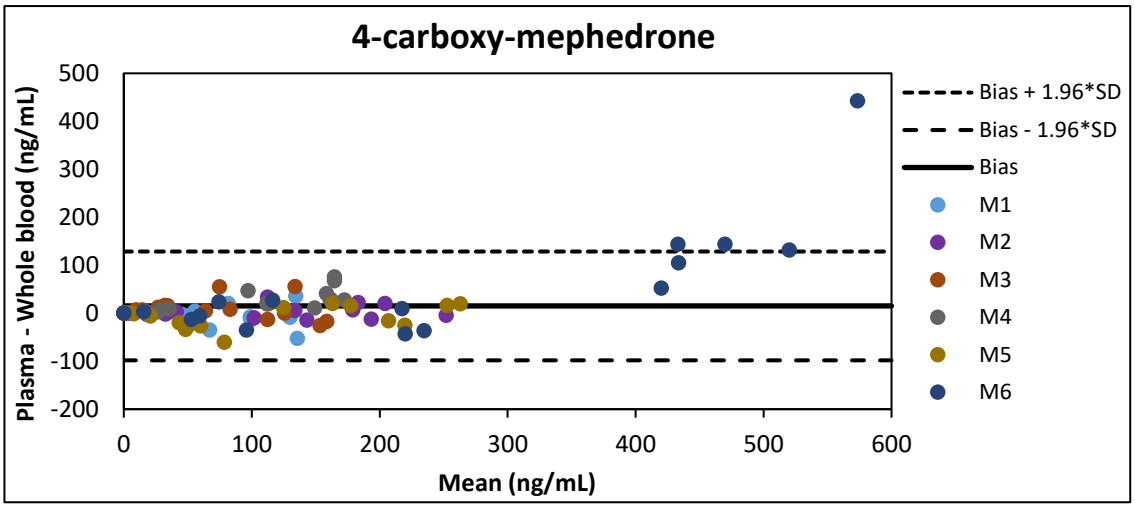


Figure 3-31. Bland-Altman analysis between plasma and whole blood for 4-carboxy-mephedrone

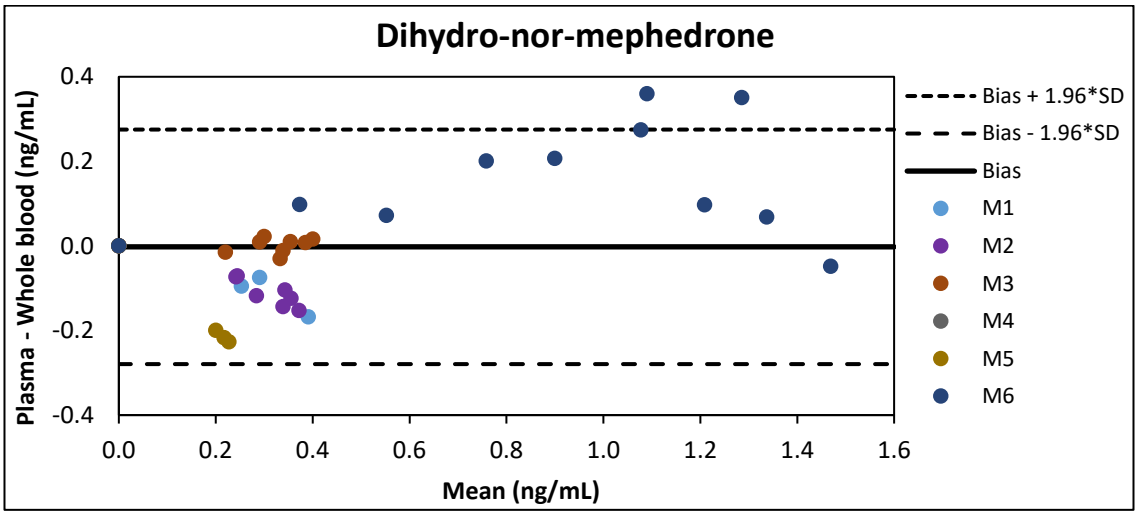


Figure 3-32. Bland-Altman analysis between plasma and whole blood for dihydro-nor-mephedrone

Bland-Altman plots show bias ± 2 SD between whole blood and plasma concentrations to be -8.8 ± 10.6 ng/mL for mephedrone, -0.37 ± 0.37 ng/mL for DHM, 1.3 ± 2.2 ng/mL for NOR, -0.05 ± 0.20 ng/mL for HYDROXY, 15.2 ± 57.9 ng/mL for 4-CARBOXY and 0.02 ± 0.13 ng/mL for DHNM. More than 95% of datapoints were within ± 2 SD of the bias for mephedrone and 4-CARBOXY whereas DHM, NOR, HYDROXY and DHNM showed poorer agreement with less than 95% of datapoints being within ± 2 SD of the bias. In addition, M6 showed notable data spread, with datapoints at higher concentrations falling outside ± 2 SD of the bias.

3.3.4.6 Pharmacokinetic analysis

Plasma drug concentrations were fitted with a single-dose, first-order elimination phase model and calculated mean pharmacokinetic parameters are summarised in Table 3-7. Individual PK data can be found in Appendix E.

Table 3-7. Mean ± SD pharmacokinetic data from analysis of mephedrone and its metabolites in plasma from 6 male participants; k_{el} and $t_{1/2}$ could not be calculated for DHNM because the elimination phase was not observed from the data

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)	CL (mL min^{-1} kg^{-1})	V (L kg^{-1})
MEPH	89.8 ± 41.7	52.5 ± 20.7	0.006 ± 0.001	1.98 ± 0.30	395 ± 144	66.5 ± 23.6	11.4 ± 4.5
DHM	1.25 ± 0.847	128 ± 37.6	0.002 ± 0.001	6.06 ± 1.66	12.6 ± 6.4	-	-
NOR	7.87 ± 5.35	130 ± 35.1	0.003 ± 0.001	4.02 ± 1.40	67.5 ± 35.9	-	-
HYDROXY	1.50 ± 1.39	75.0 ± 19.0	0.007 ± 0.003	1.96 ± 1.06	4.61 ± 3.64	-	-
4-CARBOXY	306 ± 244	70.0 ± 18.2	0.006 ± 0.002	1.94 ± 0.62	1154 ± 598	-	-
DHNM	0.609 ± 0.559	285 ± 75	-	-	8.15 ± 7.66	-	-

Mephedrone showed rapid absorption (T_{max} of 52.5 ± 20.7 min) and a relatively fast $t_{1/2}$ of 1.98 ± 0.30 h. Most mephedrone metabolites reached T_{max} shortly after the parent drug, except for NOR and DHNM which peaked later at 130 ± 35.1 min and 285 ± 75 min, respectively. 4-CARBOXY reached the highest concentration and had the largest AUC of 306 ± 244 ng/mL and 1154 ± 598 ng mL^{-1} h, respectively. Another metabolite which was present in high abundance was NOR, reaching C_{max} of 7.87 ± 5.35 ng/mL and AUC of 67.5 ± 35.9 ng mL^{-1} h. For DHNM, the observed T_{max} of 285 ± 75 min corresponded with the

C_{max} of 0.609 ± 0.559 ng/mL. No other pharmacokinetic parameters were determined for DHNM because the elimination phase was not observed from the data.

With regards to elimination, mephedrone and 4-CARBOXY showed similar kinetics, including similar $t_{1/2}$ (1.98 ± 0.30 h for mephedrone and 1.94 ± 0.62 h for 4-CARBOXY) and k_{el} (0.006 ± 0.001 min^{-1} for mephedrone and 0.006 ± 0.002 min^{-1} for 4-CARBOXY). HYDROXY was eliminated with $t_{1/2}$ of 1.96 ± 1.06 h while DHM and NOR were eliminated with $t_{1/2}$ of 6.06 ± 1.66 h and 4.02 ± 1.40 h, respectively.

3.3.4.7 Comparison of pharmacokinetic parameters between whole blood and plasma

The mean C_{max} for mephedrone and its metabolites was similar in whole blood and plasma, except for NOR which reached higher concentration in plasma (7.87 ± 5.35 ng/mL) compared to whole blood (5.12 ± 2.16 ng/mL). Mephedrone had a C_{max} of 89.8 ± 41.7 ng/mL in plasma and 101 ± 45.4 ng/mL in whole blood, DHM had a C_{max} of 1.25 ± 0.847 ng/mL in plasma and 1.45 ± 0.71 ng/mL in whole blood, HYDROXY had a C_{max} of 1.50 ± 1.39 ng/mL in plasma and 1.75 ± 1.81 ng/mL in whole blood. 4-CARBOXY reached the highest concentration in both matrices (306 ± 244 ng/mL in plasma and 241 ± 113 ng/mL in whole blood). DHNM had the lowest C_{max} in both matrices (0.609 ± 0.559 ng/mL in plasma and 0.607 ± 0.505 ng/mL in whole blood). The two major metabolites detected at high concentrations in this study correspond well with a previously published administration study where 4-CARBOXY was the most predominant metabolite detected in plasma followed by NOR [91].

The mean T_{max} of approximately 55 min for mephedrone corresponded well between whole blood and plasma, indicating rapid absorption of the drug following nasal insufflation. All other analytes had a more delayed T_{max} than mephedrone. DHNM had a T_{max} of 285 ± 75 min and 300 ± 73.5 min in plasma and whole blood, respectively, with concentrations still rising at this point in both matrices. All analytes were detectable up

to 360 min (6 h) in plasma and whole blood, with mephedrone and NOR also being detected on Day 2 in some participants.

With regards to elimination, mephedrone and 4-CARBOXY showed similar kinetics, including similar $t_{1/2}$ and k_{el} in both matrices. Mephedrone had a mean $t_{1/2}$ of 2.12 ± 0.33 h in whole blood and 1.98 ± 0.30 h in plasma whereas 4-CARBOXY had a $t_{1/2}$ of 1.70 ± 0.26 h in whole blood and 1.94 ± 0.62 h in plasma. DHM had a longer $t_{1/2}$ than mephedrone in whole blood (7.19 ± 4.19 h) and in plasma (6.06 ± 1.66 h) while NOR had a $t_{1/2}$ of 6.09 ± 2.64 h in whole blood and 4.02 ± 1.40 h in plasma. HYDROXY had a short $t_{1/2}$ of 1.52 ± 0.60 h in whole blood and 1.96 ± 1.06 h in plasma.

3.3.5 Discussion

After the intranasal administration of 100 mg of mephedrone hydrochloride, the mean C_{max} of mephedrone in plasma was 89.8 ± 41.7 ng/mL. The C_{max} was approximately 50% lower than the reported C_{max} of 179.0 ± 29.3 ng/mL following oral administration of 150 mg of mephedrone hydrochloride [91] and approximately 42% higher than the reported C_{max} of 51.7 ± 20.5 ng/mL following oral administration of 100 mg of mephedrone hydrochloride [75]. Intranasal administration of drugs results in rapid drug absorption via the nasal mucosa and avoids hepatic and intestinal first-pass metabolism. Therefore, the same dose of drug given orally and intranasally would result in faster T_{max}, higher C_{max} and larger AUC after intranasal administration. As expected, mean T_{max} in plasma was shorter in our study (0.88 ± 0.35 h) compared to the plasma T_{max} of 1 h (range: 1-2 h) reported after an oral administration of 100 mg of mephedrone hydrochloride. Moreover, the AUC in plasma calculated for up to 8 h after oral administration of 100 mg of mephedrone hydrochloride was approximately 2.5 times smaller than the AUC reported in our study following a 100 mg intranasal insufflation. Pharmacokinetic results obtained from the analysis of whole blood samples cannot be compared with the literature because, to our knowledge, this is the first study that presents this data.

In this study, mephedrone has been shown to be rapidly absorbed, reaching mean plasma T_{max} of 0.88 ± 0.35 h. This is in contrast with the plasma T_{max} reported in other controlled administration studies of MDMA (2.3 ± 1.1 h after a 100 mg oral dose) [414], methamphetamine (2.8 ± 0.1 h after a 50 mg intranasal dose) [419] and cathinone (2.31 ± 0.65 h after chewing 0.6 g of khat leaves per kg body weight for 1 h) [420]. Mephedrone also had shorter $t_{1/2}$ (1.98 ± 0.30 h) in plasma compared with MDMA (9.0 ± 2.3 h) [414] and methamphetamine (9.1 h (3-11 h)) [419]. This explains why mephedrone users report taking 100-200 mg every hour or two hours, such that they use up to 1 g or more per "session" [30,68].

The mean plasma $t_{1/2}$ of mephedrone reported in this study (2.12 ± 0.33 h in whole blood and 1.98 ± 0.30 h in plasma) corresponds well with the published $t_{1/2}$ in plasma from previous oral mephedrone administration studies (2.2 ± 0.1 h [91], 2.2 ± 0.28 h [97] and 2.2 ± 0.4 h [96]). Regarding metabolites, *Olesti et al.* [91] have reported $t_{1/2}$ of 4.5 ± 0.4 h, 5.7 ± 0.8 h and 1.94 ± 0.62 h for NOR, DHM and 4-CARBOXY in plasma, respectively, which is also in good agreement with the values reported here.

When centrifuged, whole blood can be separated into plasma and cellular components containing erythrocytes and leukocytes mixed with platelets [421]. A drug that is equally distributed between plasma and erythrocytes will give a whole blood to plasma drug distribution ratio of 1 (unity). If a drug partitions into the erythrocytes the ratio will exceed 1 and if a drug partitions into plasma the ratio will be smaller than 1 [415]. For example, THC poorly distributes into red blood cells resulting in plasma concentration being twice that of whole blood (assuming normal haematocrit of 0.45) [422]. *Huestis et al.* have developed a mathematical model for calculating cannabinoid plasma concentrations when only whole blood samples are available for analysis [423]. The model proposes multiplying whole blood concentrations by a multiplying factor of 1.6. The model was applied by *Giroud et al.* to the analysis of 8 whole blood and plasma samples from regular cannabis users [424]. The authors found whole blood to plasma distribution ratios to be about 1.6 for 3 cannabinoids and their corresponding %CV was smaller than

15.1%, suggesting plasma drug concentrations could be evaluated from whole blood concentrations when the multiplying factor of 1.6 is taken into account. Our study reports for the first-time concurrent concentrations of mephedrone and its metabolites in whole blood and plasma, allowing whole blood to plasma distribution ratios to be calculated. According to the p-values obtained from the one sample t-test (Table 3-6), median whole blood to plasma distribution ratios were statistically different from 1 (unity) for mephedrone (median: 1.11), DHM (median: 1.30) and NOR (median: 0.765). However, due to the small sample size (n=6), further studies in this area are encouraged. Calculated %CV ranged from 8.5% for mephedrone to 33.6% for NOR but was generally higher than 15%. This could be partly due to differences in haematocrit between participants, which was not determined in this study.

Inter-individual variations seen in the study might be due to genetic polymorphism of CYP2D6 which is responsible for mephedrone metabolism in humans [70]. *Olesti et al.* have demonstrated that significantly altered mephedrone plasma concentrations are a result of CYP2D6 activity, with users having no or low CYP2D6 functionality being at risk of acute toxicity [75]. Buccal swabs for CYP2D6 genotyping were collected from the participants in this study but were not analysed in time for the thesis submission.

Bland-Altman analysis showed a negative bias of -8.8 ng/mL, -0.37 ng/mL and -0.05 ng/mL for mephedrone, DHM and HYDROXY, respectively, suggesting whole blood concentrations were slightly overestimated. NOR, 4-CARBOXY and DHNM showed a positive bias of 1.25 ng/mL, 15.2 ng/mL and 0.02 ng/mL, respectively, suggesting whole blood concentrations being slightly underestimated. The agreement between methods might have also been affected by CYP2D6 polymorphism as well as plasma protein binding which influences the amount of unbound drug partitioning into the erythrocytes. This leads to an uneven distribution of a drug between plasma and the erythrocytes, which can result in concentration differences between whole blood and plasma. The extent of mephedrone or its metabolites binding to plasma proteins has not

been determined in humans but 21.6 ± 3.67% of mephedrone has been shown to bind to plasma proteins in Sprague-Dawley rats [92].

3.3.6 Conclusion

This is the first-time pharmacokinetic properties of mephedrone and its metabolites have been derived from whole blood and compared to concurrently collected plasma samples. Median whole blood to plasma distribution ratios have been shown to be statistically different from 1 (unity) for mephedrone, DHM and NOR. Inter-subject variability observed in the study might have been a result of CYP2D6 polymorphism and an unknown extent of analytes binding to plasma proteins in humans.

3.4 Dried blood spots

3.4.1 Detection of mephedrone and its metabolites in dried blood spots

Even though dried blood spots (DBS) are increasing evaluated for use in drug testing instead of venous whole blood, little is known about the distribution of mephedrone and its metabolites into this matrix. To date the only study which reported a successfully validated method for the detection of 64 NPS (including mephedrone) in DBS (collected on filter paper) was published by *Ambach et al* [376]. The method was applied to 21 authentic DBS samples (the paper does not specify where the samples were collected from) but mephedrone was not detected.

3.4.2 Dried blood spots aims

The primary aim was to investigate the distribution of mephedrone and its metabolites in dried blood spots following a controlled mephedrone administration. The secondary aim was to compare concentrations obtained in dried blood spots with those obtained in whole blood and plasma in order to assess correlation between these matrices.

3.4.3 Experimental

3.4.3.1 Reagents

Please refer to 3.1.3.1 for the information. Ten microliter Mitra® devices were purchased from Neotyrex (Torrance, USA).

3.4.3.2 Blank matrix collection

Please refer to 3.1.3.2.

3.4.3.3 Volunteer administration study and sample collection

Six healthy male volunteers nasally insufflated 100 mg of mephedrone hydrochloride supplied as a racemic mixture (purity: 96.3 ± 0.5%). Fingertips were wiped with an ethanol wipe and allowed to dry. A single-use lancet was used to prick a finger and 10 µL of capillary blood was directly collected onto the Mitra® device at -10 min (before administration), 5 min, 20 min, 60 min, 105 min, 120 min, 360 min, Day 2 and Day 3. Collected samples were then dried for 2 h at room temperature and stored desiccated at -20°C until analysis.

3.4.3.4 Working solutions

Working solutions used for the preparation of the calibration curve were made in MeOH:water (50:50 v/v) at 100, 200, 500, 1250, 2500, 5000, 12500 ng/mL for NOR and 4-CARBOXY; 50, 200, 500, 1250, 2500, 5000, 12500 ng/mL for DHM, HYDROXY, DHNM; and 200, 250, 500, 1250, 2500, 5000, 12500 ng/mL for MEPH. Working solution used for the preparation of the QC samples at low, medium and high level were made in MeOH:water (50:50 v/v) at 125, 1250, 10000 ng/mL for NOR and 4-CARBOXY; 100, 1250, 10000 ng/mL for DHM, HYDROXY, DHNM; and 250, 1250, 1000 ng/mL for MEPH. IS

solution containing MEPH-d_3 and DHM-d_3 at 20 ng/mL was prepared in 0.3% formic acid in MeOH which was used as an extraction solvent.

3.4.3.5 Calibration standards and quality control samples

Calibration standards containing NOR and 4-CARBOXY at 4, 8, 20, 50, 100, 200, 500 ng/mL; DHM, HYDROXY, DHNM at 2, 8, 20, 50, 100, 200, 500 ng/mL; and MEPH at 8, 10, 20, 50, 100, 200, 500 ng/mL were prepared by the addition of an appropriate volume of the working solution to whole blood. QC Low (5 ng/mL for NOR and 4-CARBOXY; 4 ng/mL for DHNM, HYDROXY, DHM and 10 ng/mL for MEPH), QC Med (50 ng/mL for all analytes) and QC High (400 ng/mL for all analytes) were prepared by the addition of an appropriate volume of the working solution to whole blood.

Calibration standards and QCs were prepared fresh on the day of sample analysis. Blanks containing whole blood but no IS and one sample containing whole blood and IS were also prepared and taken through the extraction.

3.4.3.6 Sample preparation

Dried tips from the Mitra® devices were transferred to Eppendorf tubes to which 0.3% formic acid in MeOH containing IS was added. 0.3% formic acid in MeOH which did not contain the IS was added to the blanks. All samples were sonicated at 35 kHz for 15 min and vortex mixed for 5 min at 1300 rpm. The solvent was vacuum evaporated at 45°C and samples were reconstituted with 100 µL of 0.1% formic acid in ACN:water (10:90 v/v). Samples where visible precipitate has formed were centrifuged for 5 min at 10,000 rpm.

Due to the nature of the collected samples, dilution could not be performed at the beginning of an extraction. As a result, 1 in 5 dilution was performed after sample reconstitution (see Section 2.6.6.7 in Chapter 2 for more details). Where dilution was required, 3 additional QCs were extracted and diluted in the same manner.

3.4.3.7 LC-MS/MS conditions

DHM-d_3 was used as an IS for 4-CARBOXY. For other details please refer to 3.1.3.7.

3.4.3.8 Pharmacokinetic calculations

Please refer to Section 2.3 in Chapter 2.

3.4.3.9 Validation procedure

Please refer to Section 2.5 in Chapter 2.

3.4.4 Results

3.4.4.1 Method validation

Please refer to Section 2.6.6 in Chapter 2.

3.4.4.2 Concentrations of mephedrone and its metabolites in dried blood spots

Mean DBS concentrations ± SD for mephedrone, NOR and 4-CARBOXY in all 6 participants are presented in Figure 3-33 - Figure 3-36. The remaining analytes were only detected in a handful of samples. DHNM was quantified in M4 at 20 min (3.26 ng/mL). DHM was detected between 60 min and 120 min in M3 (2.10-3.17 ng/mL) and in M6 (2.06-2.55 ng/mL). HYDROXY was present in M2 at 60 min (2.46 ng/mL), 120 min (2.23 ng/mL) and 360 min (2.57 ng/mL) as well as in M4 at 60 min (2.63 ng/mL) and in M6 at 150 min (2.01 ng/mL).

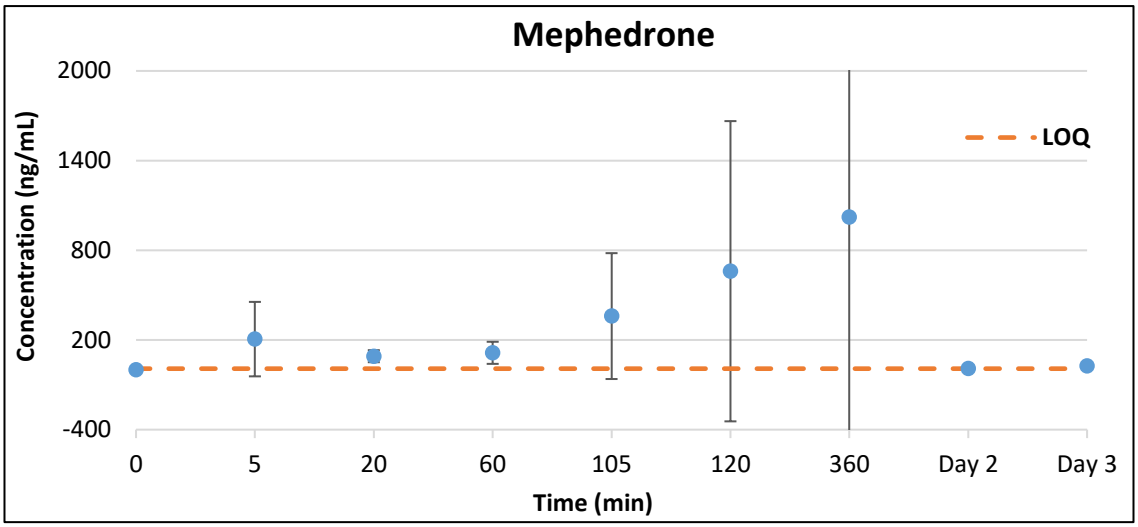


Figure 3-33. Mean mephedrone concentrations ± SD in DBS (n=6); the orange line shows the LOQ; the error bar at 360 min is not shown in its entirety for clarity (it extends from -1400 ng/mL to 3500 ng/mL)

Figure 3-33 shows the C_{max} at 360 min which is unlikely to occur so late for mephedrone. A closer look at the data reveals that the C_{max} occurs at 360 min because the concentration detected in the 360 min sample in M4 was 5,782 ng/mL, roughly 5 times higher than any other detected concentration at this timepoint. When that datapoint was excluded from the data set, mephedrone concentration peaked at 120 min (660 ± 1,004 ng/mL) and dropped to 69.9 ± 45.0 ng/mL at 360 min (Figure 3-34).

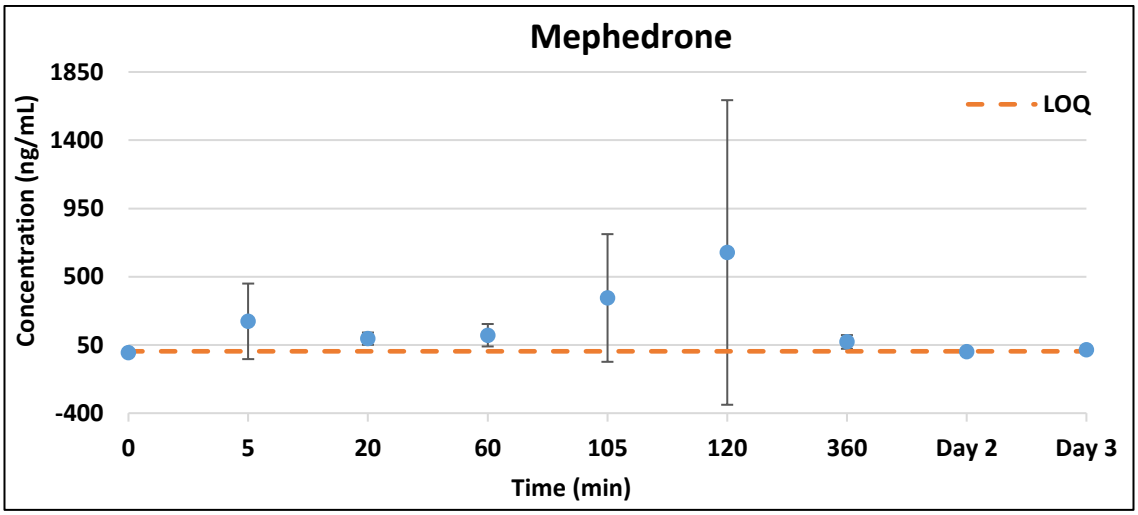


Figure 3-34. Mean mephedrone concentrations ± SD in DBS (n=6 but with the 360 min datapoint from M4 removed); the orange line shows the LOQ

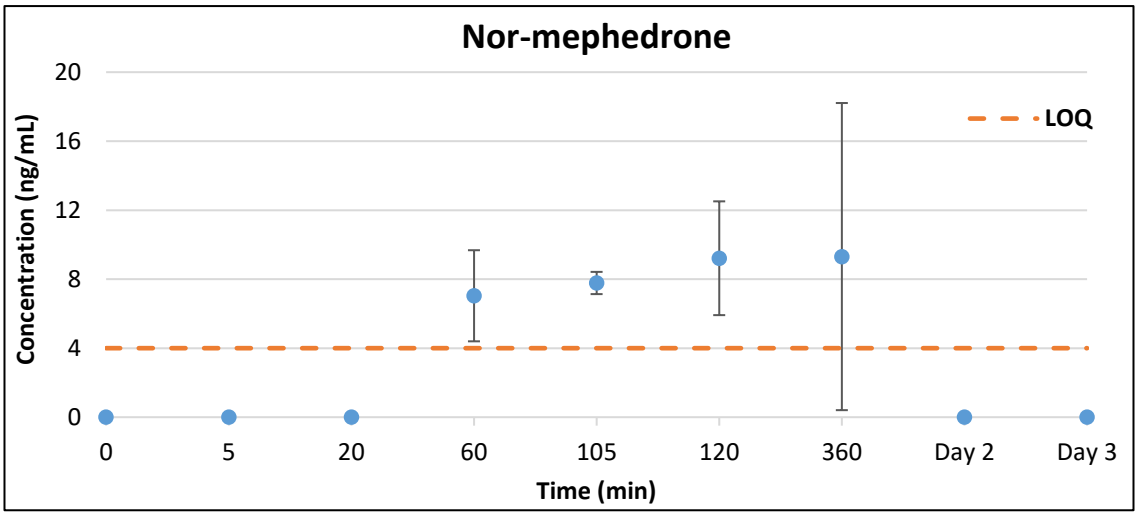


Figure 3-35. Mean nor-mephedrone concentrations ± SD in DBS (n=6); the orange line shows the LOQ

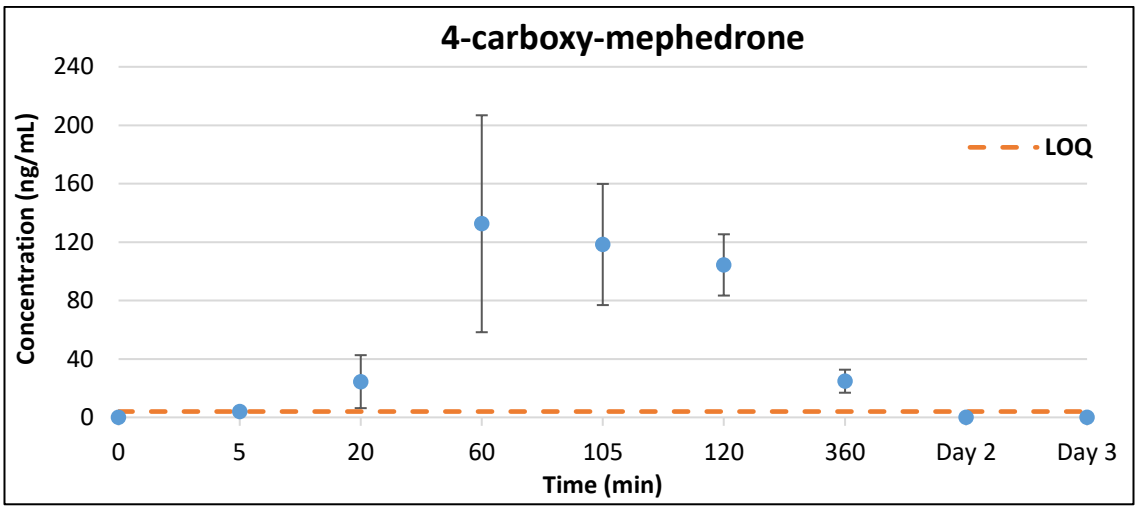


Figure 3-36. Mean 4-carboxy-mephedrone concentrations ± SD in DBS (n=6); the orange line shows the LOQ

3.4.4.3 Comparison of analyte concentrations in dried blood spot and whole blood

Mephedrone, NOR and 4-CARBOXY were detectable in all participants in whole blood and DBS. Mephedrone was detected at the first collection timepoint (5 min) in both matrices. NOR first appeared in whole blood in all participants after 20 min but was

detected at 60 min in M1, M2, M3 and M6 in DBS. 4-CARBOXY was detected after 5 min in M6 and after 20 min in M1-M5 in both matrices. As shown in Figure 3-37 - Figure 3-39, NOR and mephedrone reached higher mean concentration in DBS whereas 4-CARBOXY was roughly two times more abundant in whole blood.

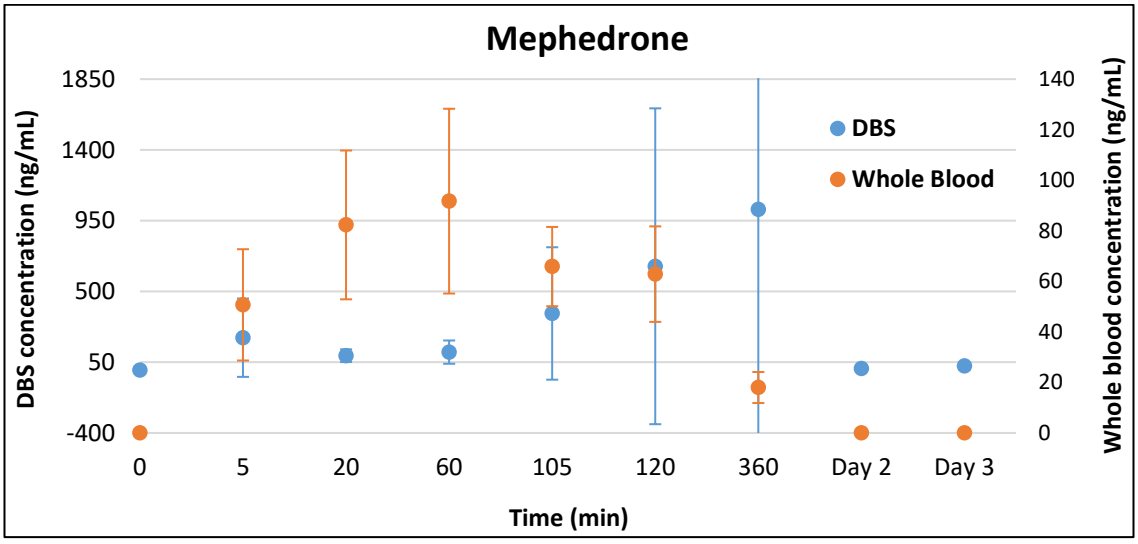


Figure 3-37. Mean mephedrone concentrations ± SD in DBS and whole blood (n=6); note that whole blood concentrations are shown on the secondary axis on the right-hand side and the entirety of the error bar at 360 min is not shown for clarity

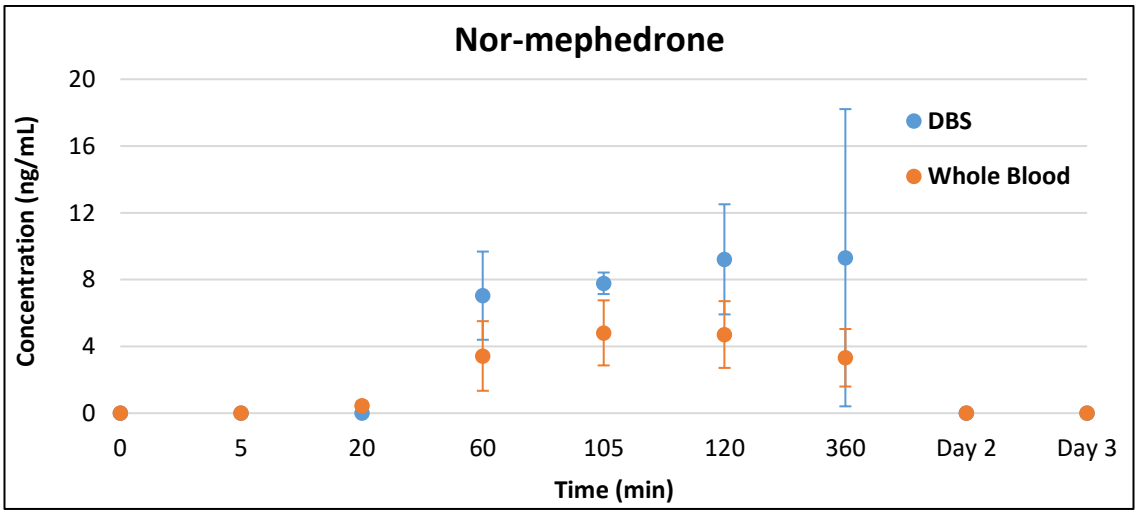


Figure 3-38. Mean nor-mephedrone concentrations ± SD in DBS and whole blood (n=6)

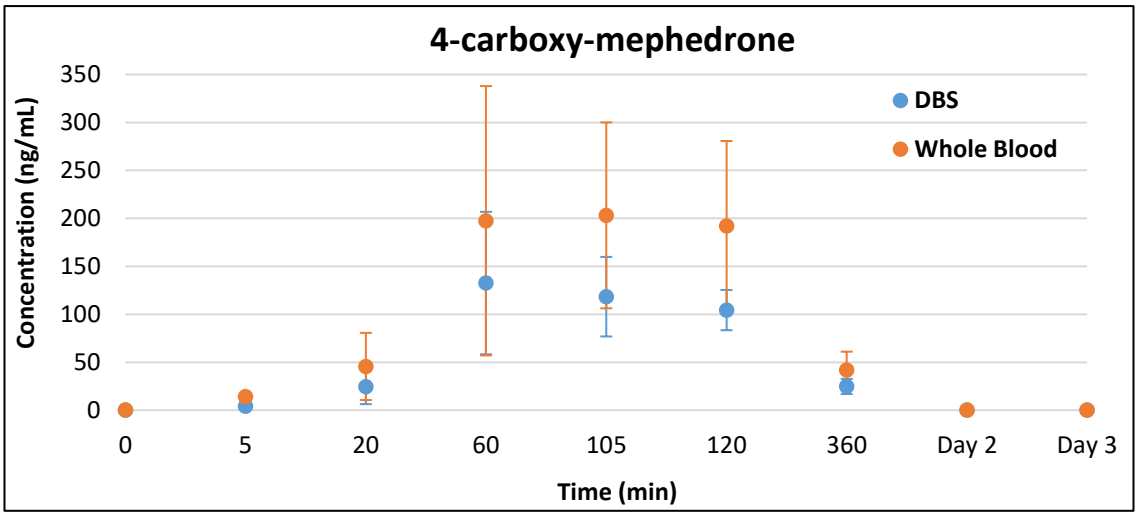


Figure 3-39. Mean 4-carboxy-mephedrone concentrations ± SD in DBS and whole blood (n=6)

3.4.4.4 Comparison of analyte concentrations in dried blood spots and plasma

Mephedrone, NOR and 4-CARBOXY were detectable in all participants in plasma and DBS. Mephedrone was detected at the first collection timepoint (5 min) in both matrices. NOR first appeared in plasma after 20 min in all participants except for M6 where it was detected after 5 min. In DBS NOR was detected after 60 min in M1, M2, M3 and M6 as well as after 105 min in M4. 4-CARBOXY was detected after 20 min in M1-M5 and after 5 min in M6 in DBS. In plasma, 4-CARBOXY was present in the samples after 5 min in M3-M6 and after 20 min in M1 and M2. As shown in Figure 3-40 - Figure 3-42, NOR and mephedrone reached higher mean concentration in DBS whereas 4-CARBOXY was roughly two times more abundant in plasma.

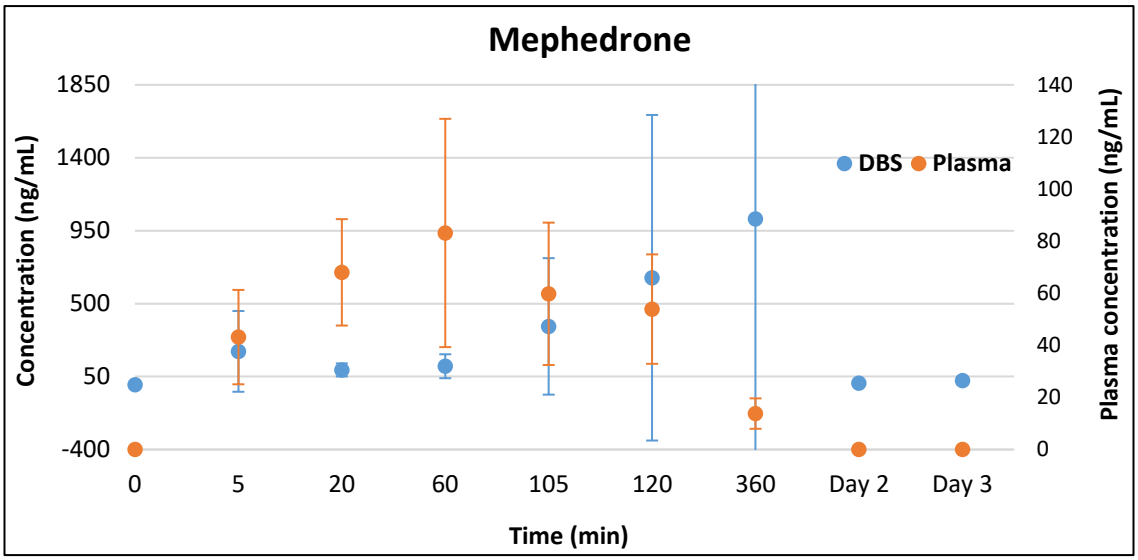


Figure 3-40. Mean mephedrone concentrations ± SD in DBS and plasma (n=6); note that plasma concentrations are shown on the secondary axis on the right-hand side and the entirety of the error bar at 360 min is not shown for clarity

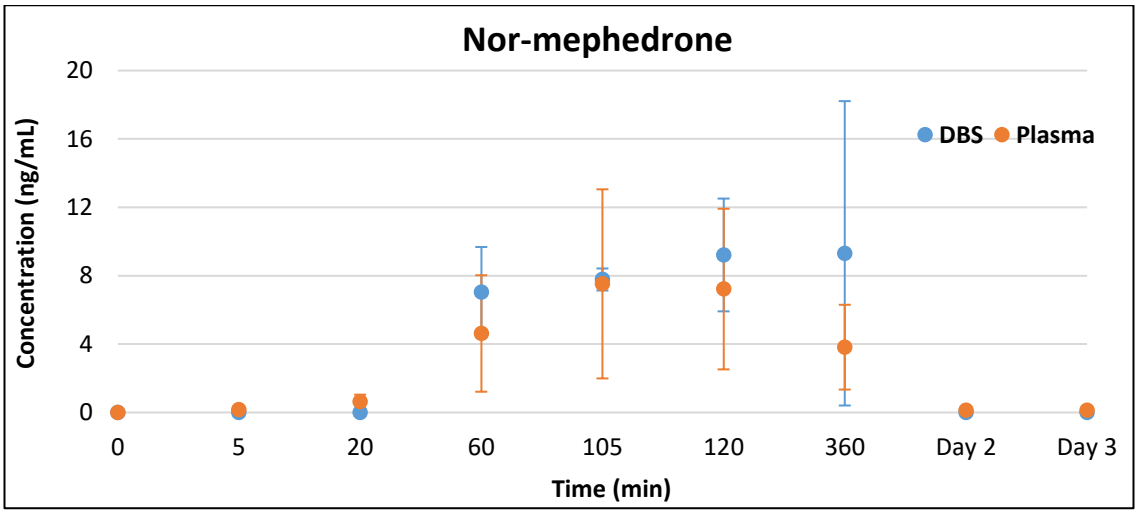


Figure 3-41. Mean nor-mephedrone concentrations ± SD in DBS and plasma (n=6)

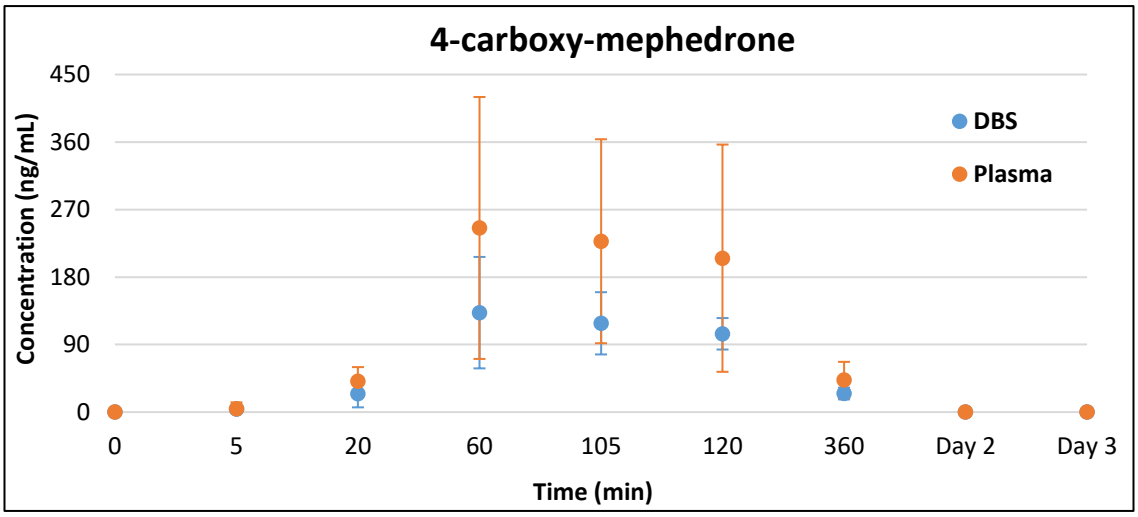


Figure 3-42. Mean 4-carboxy-mephedrone concentrations ± SD in DBS and plasma (n=6)

3.4.4.5 Correlation with whole blood

Correlation between analyte concentrations in whole blood and DBS for each participant is presented in Figure 3-43 - Figure 3-45 (the black dotted line shows a trend line). Individual correlation can be found in Appendix E.

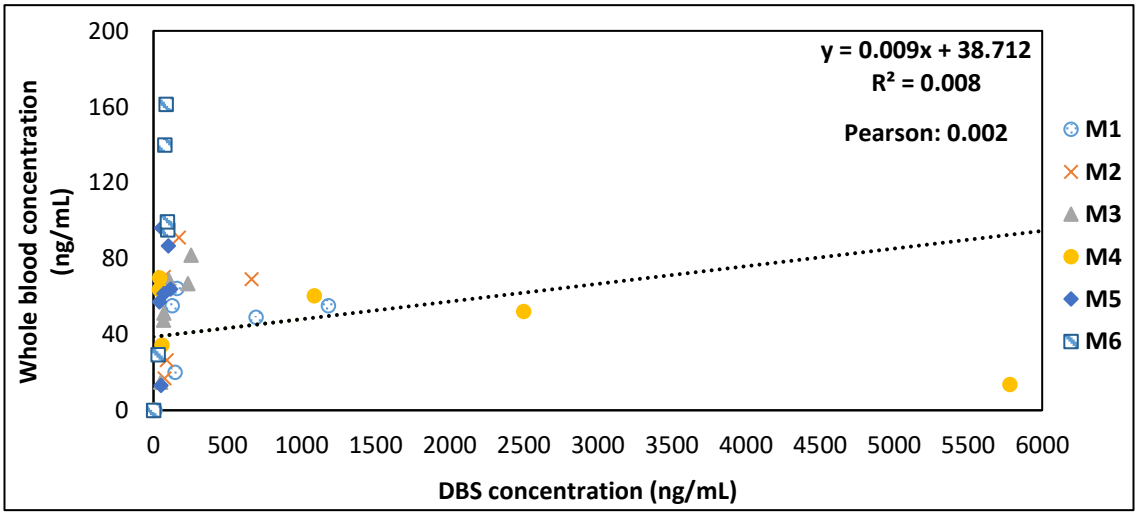


Figure 3-43. Correlation between DBS and whole blood concentrations for mephedrone

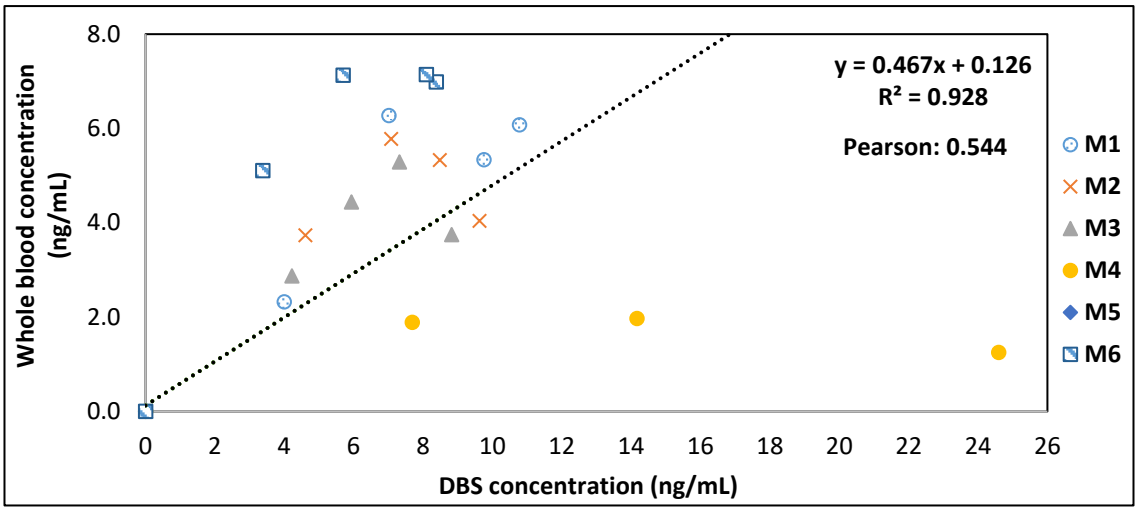


Figure 3-44. Correlation between DBS and whole blood concentrations for nor-mephedrone

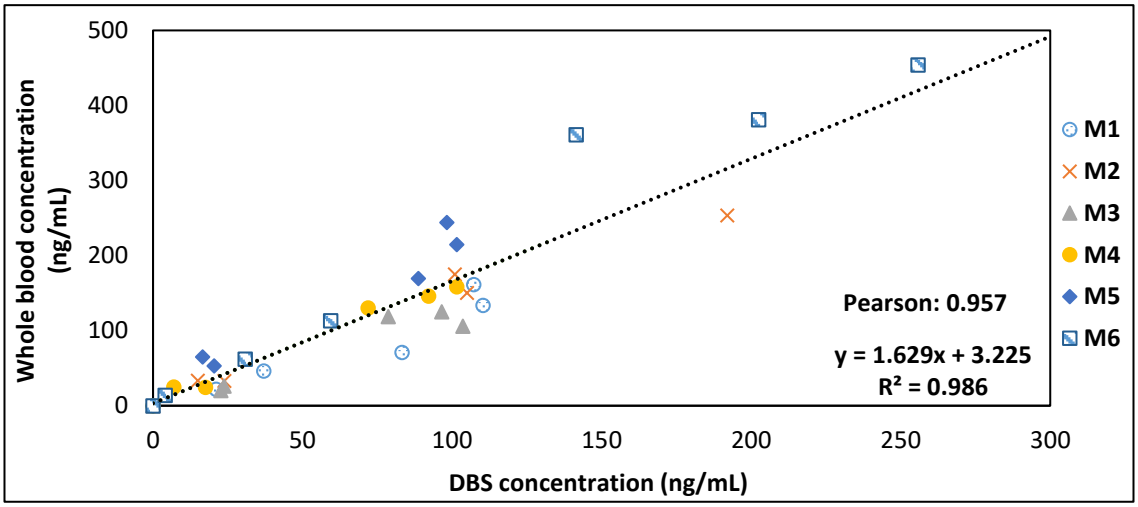


Figure 3-45. Correlation between DBS and whole blood concentrations for 4-carboxy-mephedrone

Mephedrone did not correlate well between the two matrices as evidenced by the Pearson correlation coefficient of 0.002. NOR and 4-CARBOXY showed better correlation with the Pearson correlation coefficient of 0.544 and 0.957, respectively.

3.4.4.6 Correlation with plasma

Correlation between analyte concentrations in plasma and DBS for each participant is presented in Figure 3-46 - Figure 3-48 (the black dotted line shows a trend line). Individual correlation can be found in Appendix E.

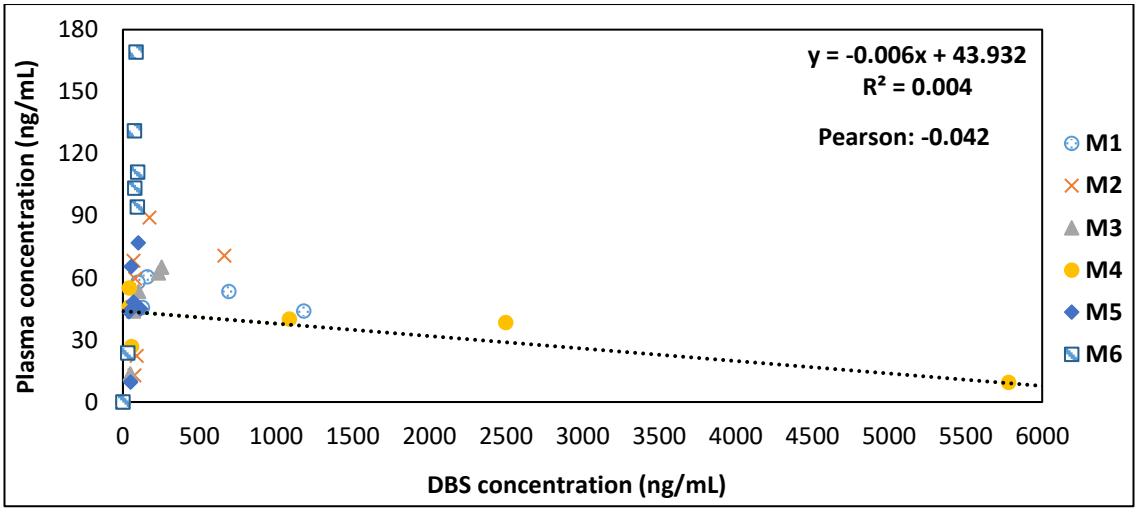


Figure 3-46. Correlation between DBS and plasma concentrations for mephedrone

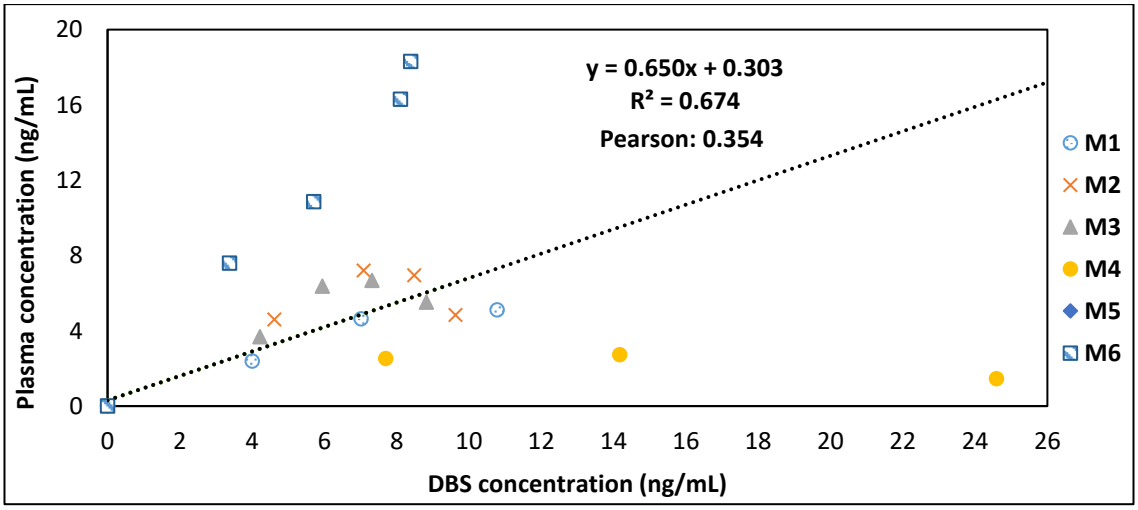


Figure 3-47. Correlation between DBS and plasma concentrations for nor-mephedrone

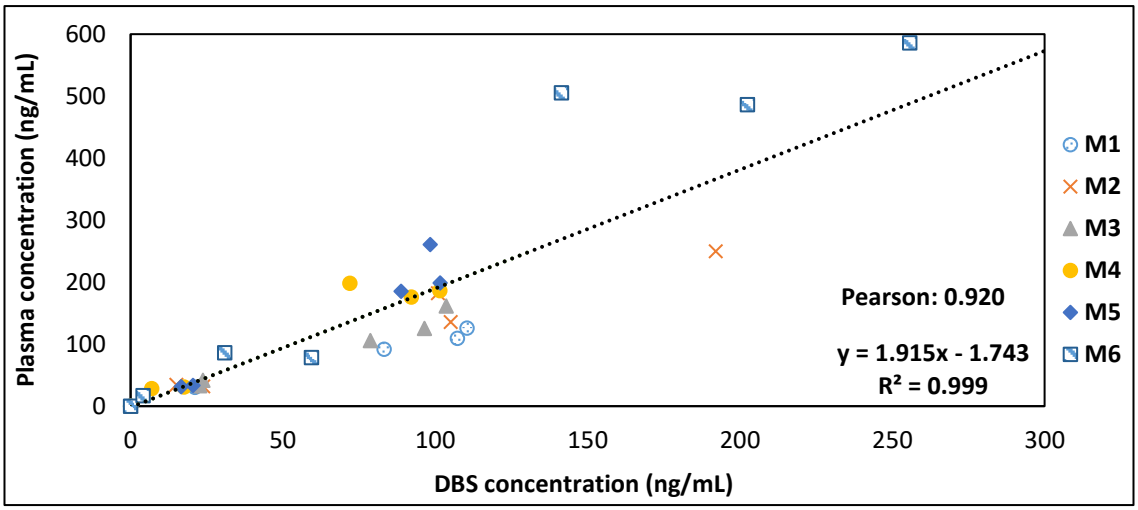


Figure 3-48. Correlation between DBS and plasma concentrations for 4-carboxy-mephedrone

Mephedrone and NOR did not correlate well between the two matrices as evidenced by the Pearson correlation coefficient of -0.042 and 0.354, respectively. 4-CARBOXY showed a much better correlation with the Pearson correlation coefficient of 0.920.

3.4.4.7 Whole blood and dried blood spots - method comparison

Bland-Altman analysis was performed to examine the agreement between concentrations obtained in whole blood and DBS. Bland-Altman plots, presented in Figure 3-49 - Figure 3-51, show the difference between paired concentrations from DBS and whole blood samples plotted against the mean of the two concentrations calculated for each individual sample. Dotted lines represent the 95% limits of agreement (mean difference ± 2 SD).

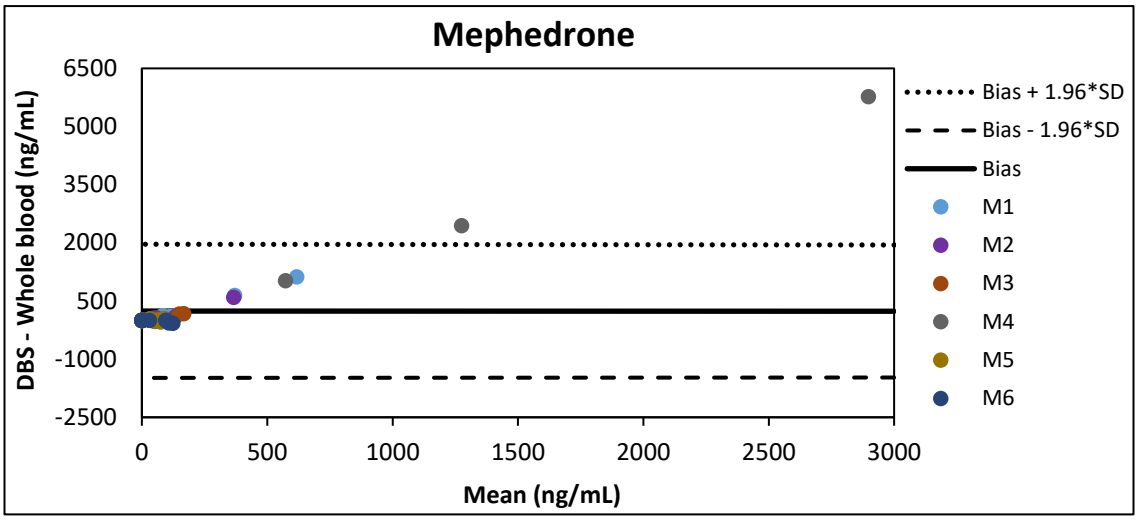


Figure 3-49. Bland-Altman plots comparing whole blood and DBS methods for mephedrone

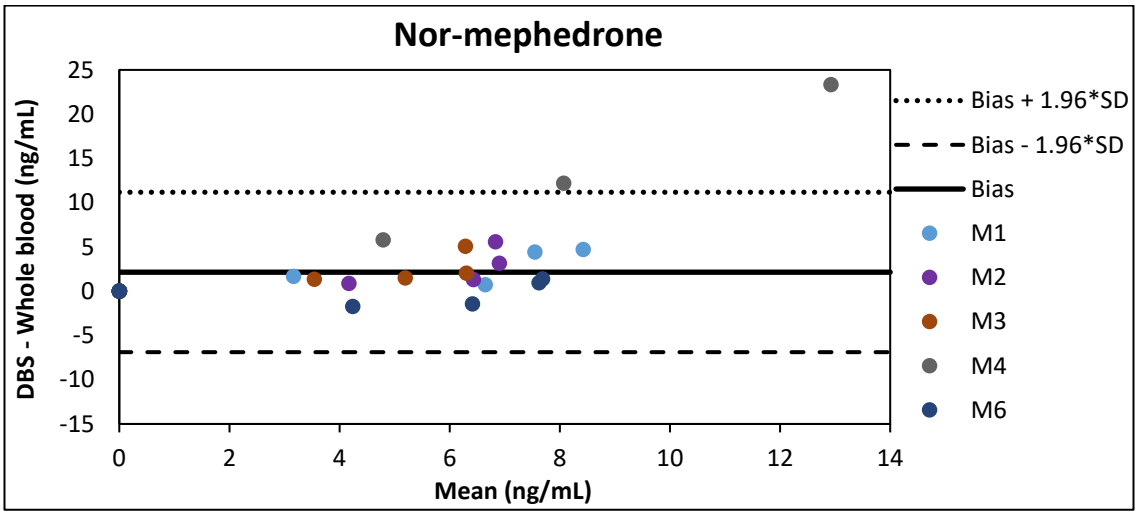


Figure 3-50. Bland-Altman plots comparing whole blood and DBS methods for nor-mephedrone

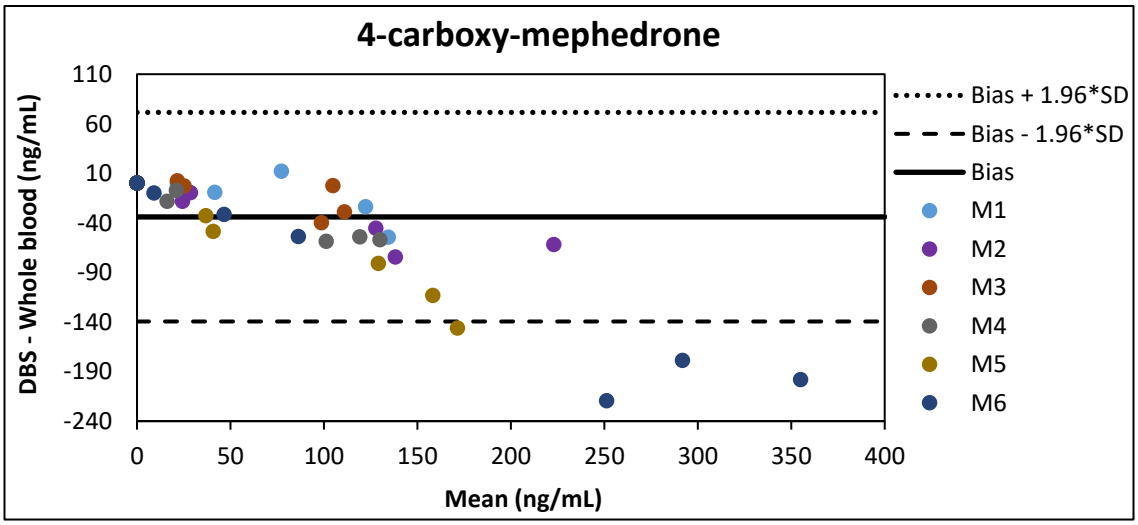


Figure 3-51. Bland-Altman plots comparing whole blood and DBS methods for 4-carboxy-mephedrone

Bland-Altman plots showed bias ± SD between whole blood and DBS concentrations to be 235 ± 872 ng/mL for mephedrone, 2.15 ± 4.61 ng/mL for NOR and -34.0 ± 53.8 ng/mL for 4-CARBOXY. Whole blood mephedrone concentrations were in good agreement with DBS concentrations with more than 95% of datapoints being within ± 2 SD of the bias. The agreement was poorer for NOR and 4-CARBOXY where 94.1% and 91.8% of datapoints, respectively, were found to be within ± 2 SD of the bias.

3.4.4.8 Plasma and dried blood spots - method comparison

Bland-Altman analysis was performed to examine the agreement between concentrations obtained in plasma and DBS. Bland-Altman plots, presented in Figure 3-52 - Figure 3-54, show the difference between paired concentrations from DBS and plasma samples plotted against the mean of the two concentrations calculated for each individual sample. Dotted lines represent the 95% limits of agreement (mean difference ± 2 SD).

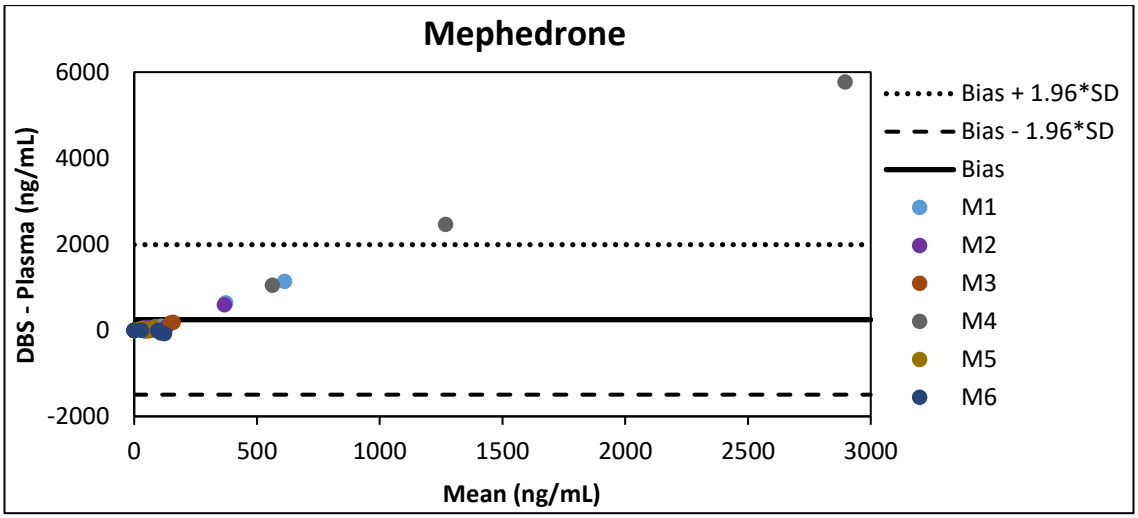


Figure 3-52. Bland-Altman plots comparing plasma and DBS methods for mephedrone

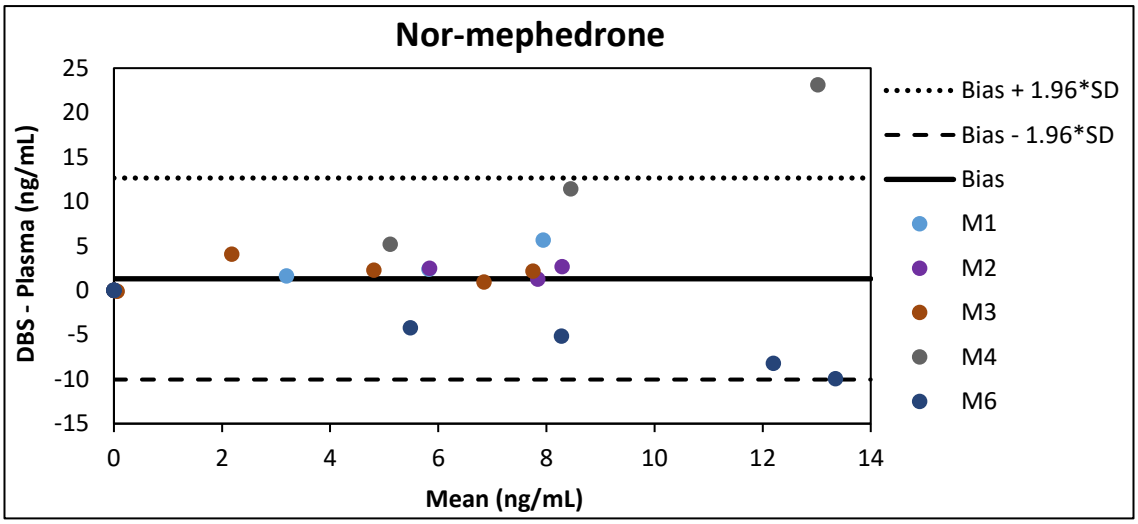


Figure 3-53. Bland-Altman plots comparing plasma and DBS methods for nor-mephedrone

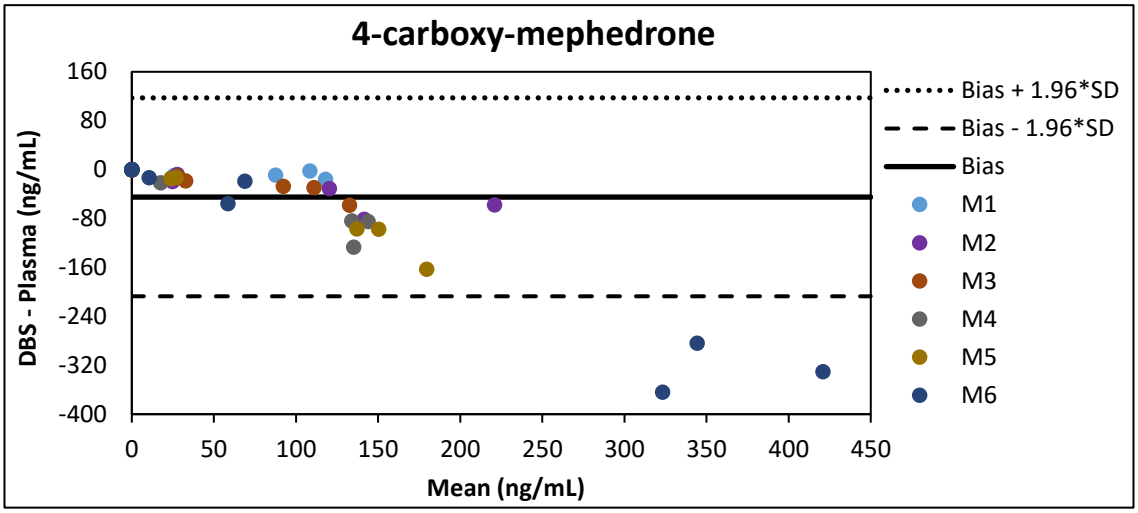


Figure 3-54. Bland-Altman plots comparing plasma and DBS methods for 4-carboxy-mephedrone

Bland-Altman plots showed bias ± SD between plasma and DBS concentrations to be 247 ± 889 ng/mL for mephedrone, 1.30 ± 5.78 ng/mL for NOR and -44.9 ± 82.8 ng/mL for 4-CARBOXY. Concentration of mephedrone and NOR in plasma was in good agreement with DBS concentrations where more than 95% of datapoints were within ± 2 SD of the bias. The agreement was poorer for 4-CARBOXY where 93.8% of datapoints were found to be within ± 2 SD of the bias.

3.4.4.9 Pharmacokinetic analysis

DBS drug concentrations were fitted with a single-dose, first-order elimination phase model and calculated mean pharmacokinetic parameters are summarised in Table 3-8. For mephedrone and NOR pharmacokinetic parameters were only calculated from 3 participants (M2, M3 and M6) while the data for 4-CARBOXY is based on 5 participants (M1, M2, M3, M4, M6). This is because insufficient number of samples was collected in the elimination phase or the log-linear regression did not produce R^2 of at least 0.970.

Table 3-8. Mean ± SD pharmacokinetic data from analysis of mephedrone and its metabolites in dried blood spots

** From 3 male participants (mean C_{max}, T_{max}, AUC is based on 6 participants)*

*** From 5 male participants (mean C_{max}, T_{max}, AUC is based on 6 participants)*

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)
MEPH *	1345 ± 2213	143 ± 108	0.002 ± 0.002	6.83 ± 4.02	13065 ± 27719
NOR *	12.4 ± 6.85	141 ± 125	0.003 ± 0.001	4.29 ± 0.94	130 ± 102
4-CARBOXY **	144 ± 65	85.0 ± 27.9	0.006 ± 0.001	1.98 ± 0.44	658 ± 175

Due to the small sample size (n=3), large standard deviation was associated with the C_{max}, T_{max}, k_{el}, $t_{1/2}$ and AUC obtained for mephedrone, making the results unreliable. Pharmacokinetic parameters associated with NOR and 4-CARBOXY did not show large variability and will be discussed here. NOR reached a C_{max} of 12.4 ± 6.85 ng/mL at 141 ± 125 min which was later than 4-CARBOXY which peaked at 85.0 ± 27.9 min (C_{max} of 144 ± 65 ng/mL). Moreover, 4-CARBOXY had larger AUC of 658 ± 175 ng mL^{-1} h compared to NOR (AUC of 130 ± 102 ng mL^{-1} h). With regards to elimination, 4-CARBOXY had a $t_{1/2}$ of 1.98 ± 0.44 h (k_{el} of 0.006 ± 0.001 min^{-1}) while NOR was eliminated with $t_{1/2}$ of 4.29 ± 0.94 h (k_{el} of 0.003 ± 0.001 min^{-1}).

3.4.4.10 Comparison of pharmacokinetic parameters between dried blood spots, whole blood and plasma

Mephedrone and five Phase I metabolites were detected in whole blood and plasma but only mephedrone, NOR and 4-CARBOXY were detected in DBS. As mentioned in 3.4.4.9, pharmacokinetic data associated with mephedrone in DBS had large standard deviation and could not be reliably compared with whole blood and plasma.

NOR and 4-CARBOXY displayed similar T_{max} between DBS, whole blood and plasma. NOR had a T_{max} of 130 ± 35 min in plasma, 133 ± 37.5 min in whole blood and 141 ± 125 min in DBS. 4-CARBOXY peaked at 70.0 ± 18.2 min in plasma, 85.0 ± 24.5 min in whole blood and 85.0 ± 27.9 min in DBS. NOR had a $t_{1/2}$ of 6.09 ± 2.64 h and 4.02 ± 1.40 h in whole blood and plasma, respectively. Mean $t_{1/2}$ of 4.29 ± 0.94 h in DBS corresponded well with the value in plasma but was smaller than the $t_{1/2}$ in whole blood.

Interestingly, mean C_{max} obtained for NOR was higher in DBS whereas mean C_{max} obtained for 4-CARBOXY was higher in whole blood and plasma. These findings correspond well with the concentrations profiles presented in Figure 3-37 - Figure 3-39 for whole blood and in Figure 3-40 - Figure 3-42 for plasma where NOR reached higher concentrations in DBS while 4-CARBOXY reached higher concentrations in plasma/whole blood at all timepoints.

3.4.5 Discussion

Lipophilic drugs can readily cross capillary membranes while hydrophilic drugs do it more slowly at a rate that is inversely proportional to their molecular mass. 4-CARBOXY, being a more polar metabolite than NOR is, therefore, likely to be mostly confined to venous blood. This may explain why 4-CARBOXY reached lower concentrations in DBS compared to whole blood and plasma. On the other hand, NOR is a more lipophilic metabolite than 4-CARBOXY. Mean concentrations of NOR in DBS were higher than the

concentrations measured in whole blood and plasma. It has been hypothesised that this may be due to the concentration difference between capillary and venous blood. Capillary blood collected from a finger prick is a mixture of arterial and venous blood, but it also contains interstitial and intracellular fluids. Capillary blood is generally said to resemble arterial blood more than venous blood [425]. Significant differences between capillary and venous paracetamol concentrations have been previously reported during the absorption phase (up to 1 h post oral administration) [283], suggesting that an equilibrium throughout the blood and body fluids needs to be achieved for reliable comparison. Higher concentrations of artemisinin (up to 500 min after administration) [426] and tacrolimus [425] in capillary blood have also been reported but authors found it difficult to explain. The concentration difference may be attributed to the change in the glycation state of haemoglobin in dried blood possibly causing extraction bias [427], but this requires further studies.

The first sample collected at 5 min from all participants showed high mephedrone concentrations, some of which reached the highest concentration across all collected samples. This was, however, not the case for the metabolites (4-CARBOXY and NOR) which suggests that samples must have become contaminated with mephedrone only. Even though participants nasally insufflated mephedrone in a separate room, wore gloves and cleaned their hands before and after drug administration, it is possible that they used fingers to wipe their nose after the intranasal mephedrone insufflation, inadvertently picking up residual mephedrone powder which was not effectively removed by cleaning fingertips with an ethanol wipe. Samples can also be prone to contamination during extraction, but mephedrone was not present in the blank samples extracted alongside DBS samples. Even though contamination was observed in samples collected at 5 min, it is unlikely to have an impact on DBS collection in real life where samples would not be collected so quickly after mephedrone use.

The Pearson correlation coefficient showed very strong correlation between 4-CARBOXY DBS concentrations and both whole blood (Pearson = 0.957) and plasma (Pearson =

0.920) concentrations. All other analytes demonstrated poor to moderate correlation. NOR showed good correlation between DBS and whole blood (Pearson = 0.544) but was poorly correlated between DBS and plasma (Pearson = 0.354). Poor agreement was also observed for mephedrone between DBS and whole blood (Pearson = 0.002) as well as plasma (Pearson = -0.042), which might be related to differences in concentrations between capillary and venous blood. Bland-Altman analysis showed that more than 95% of the observed concentrations were within ± 2 SD of the bias for mephedrone and NOR in plasma and within ± 2 SD of the bias for mephedrone in whole blood. Negative bias of -34.0 ng/mL and -44.9 ng/mL was observed for 4-CARBOXY in whole blood and plasma, respectively, suggesting DBS concentrations were overestimated. Mephedrone and NOR showed positive bias in whole blood (235 ng/mL and 2.15 ng/mL, respectively) and plasma (247 ng/mL and 1.30 ng/mL, respectively), suggesting DBS concentrations were underestimated. No trend in variation over the concentration range was observed.

3.4.6 Conclusion

This is the first-time mephedrone and two of its metabolites (NOR and 4-CARBOXY) were detected in DBS collected from a controlled administration study. Even though the strongest correlation was reported for 4-CARBOXY between DBS and whole blood/plasma concentrations, Bland-Altman analysis showed wide confidence intervals demonstrating poor agreement between the methods. However, the agreement might have been affected by the differences in concentrations between capillary and venous blood.

3.5 Pharmacodynamics

3.5.1 Pharmacodynamics aims

The aim was to analyse the responses to the visual analogue scale (VAS), which measures the changes in the subjective effects following drug intake. The secondary aim

was to investigate changes in the psychological effects (heart rate, blood pressure, body temperature, blood oxygen saturation) after mephedrone administration.

3.5.2 Experimental details

To determine whether participants were experiencing neuropsychiatric effects of mephedrone, participants were asked a series of questions using a validated score incorporating a VAS. This was done at -10 min (before mephedrone administration) and then at every 15 min from the time of mephedrone administration until 2 h and then at 2.5 h, 3 h, 5 h and 6 h [386]. VAS assessed subjective experience of: "drug effect, bad effect, good drug effect, high, stimulated, sad, confused, fearful, liking, dizzy, experiencing changes in distance, light, hearing, body sensation, surrounding". VAS was presented as 10 cm horizontal lines, where the 0 cm point indicated no effects/changes and the 10 cm point indicated extreme effects/changes. VAS used in the administration study can be found in Appendix D.

In addition, each participant had their baseline observations (heart rate, blood pressure, body temperature, blood oxygen saturation) checked before mephedrone administration and then continually monitored and recorded at each point of sampling or every 20 min (whichever was shorter) after mephedrone administration until 6 h.

3.5.3 Results

As shown in Table 3-9, mephedrone administration caused mean increases in all VAS parameters, except for "bad drug effects" and "sadness" which were not reported. Most subjective effects commenced after 15 min, except for "changes in light" which was reported after 30 min. All subjective effects other than "changes in distance" and "changes in light" also peaked at 15 min and returned to baseline levels after 60-300 min. Individual data can be found in Appendix E.

Table 3-9. Changes in VAS parameters, expressed as cm on the VAS scale, after internasal mephedrone administration

VAS parameters	First effects (cm ± SD, min)	Peak (cm ± SD, min)	Return to baseline (min)
Drug effect	6.5 ± 3.5, 15	6.5 ± 3.5, 15	150
Bad drug effect	Not reported	Not reported	Not reported
Good drug effect	5.6 ± 4.2, 15	5.6 ± 4.2, 15	300
High	5.7 ± 3.5, 15	5.7 ± 3.5, 15	180
Stimulated	5.8 ± 2.5, 15	5.8 ± 2.5, 15	120
Sadness	Not reported	Not reported	Not reported
Confused	2.1 ± 2.2, 15	2.1 ± 2.2, 15	150
Fearful	0.58 ± 0.58, 15	0.58 ± 0.58, 15	60
Liking	6.5 ± 2.9, 15	6.5 ± 2.9, 15	120
Dizzy	2.8 ± 1.8, 15	2.8 ± 1.8, 15	75
Changes in distance	0.58 ± 0.66, 15	0.75 ± 1.2, 45	75
Changes in light	0.33 ± 0.52, 30	0.58 ± 1.4, 60	90
Changes in hearing	1.1 ± 1.7, 15	1.1 ± 1.7, 15	75
Changes in body sensation	5.3 ± 2.6, 15	5.3 ± 2.6, 15	90
Changes in surrounding	1.2 ± 1.3, 15	1.2 ± 1.3, 15	75

Detailed changes over time in VAS “drug effects”, “high” and “liking” are presented for each participant in Figure 3-55 - Figure 3-57 (errors are omitted for clarity but are listed in Table 3-9). While VAS “drug effects”, “high” and “liking” declined after peaking for M1-M5, M6 experienced longer effects (10 cm on the VAS scale) which lasted 60 min (“drug effects”, “high”) and 75 min (“liking”) after peaking.

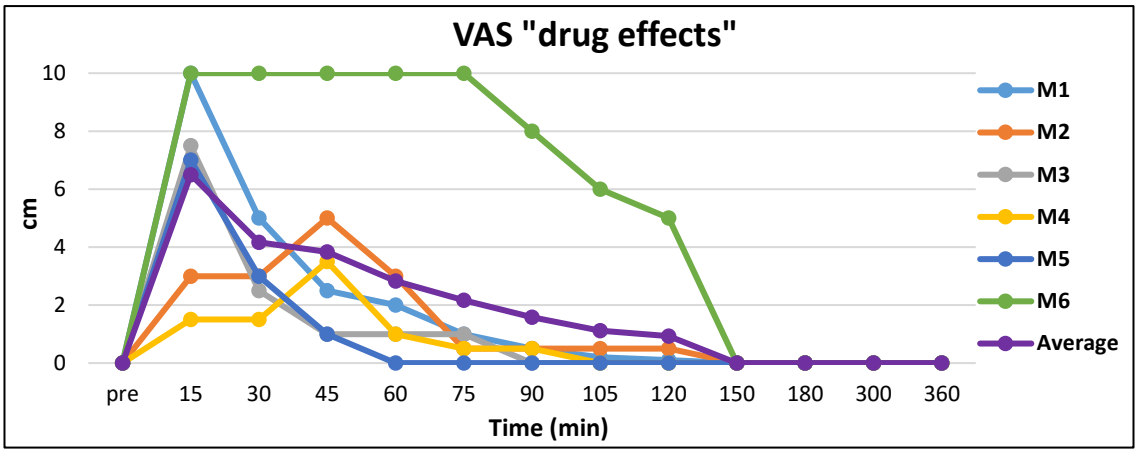


Figure 3-55. c

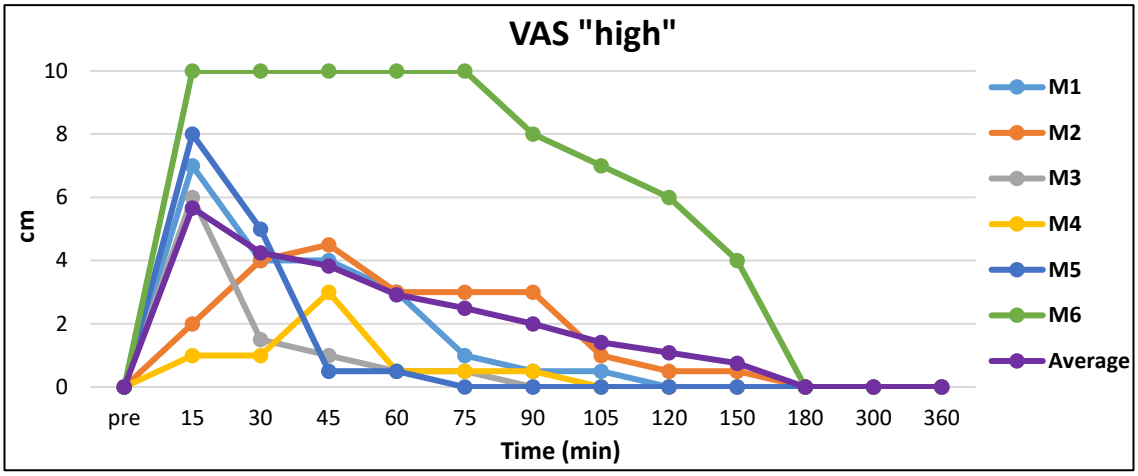


Figure 3-56. Changes in VAS "high" over time in each participant on a 0-10 cm scale

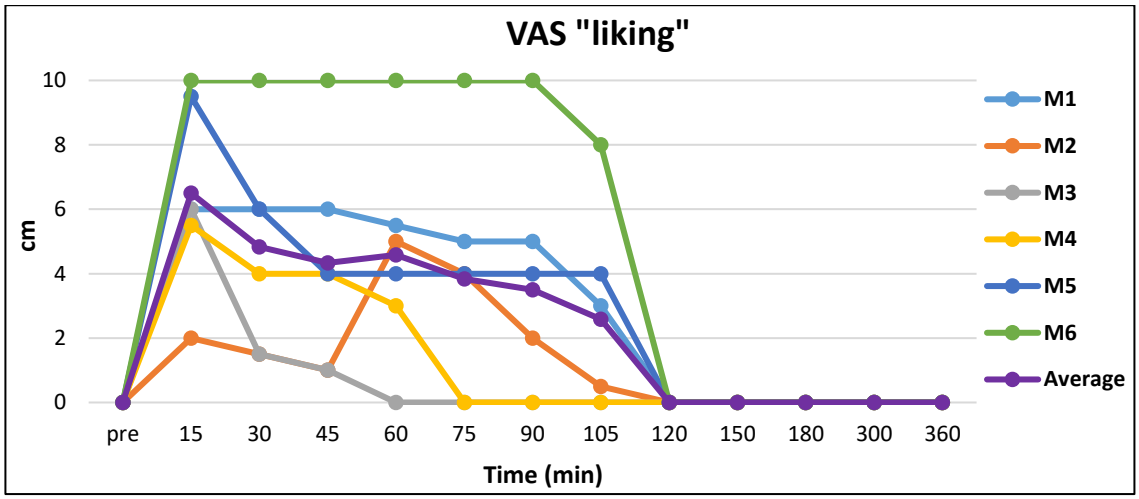


Figure 3-57. Changes in VAS "liking" over time in each participant on a 0-10 cm scale

Mephedrone administration was also associated with changes in heart rate, systolic blood pressure (SBP) and diastolic blood pressure (DBP). As shown in Table 3-10, SBP and DBP increased from the baseline level by 29 ± 13 mmHg and 18 ± 5 mmHg, respectively. SBP peaked (155 ± 17 mmHg) after 7.5 ± 4.2 min and lasted until 102 ± 54 min, representing an 11-35% increase above the pre-dose levels. DBP peaked (95 ± 9 mmHg) after 16 ± 15 min and lasted until 93 ± 67 min, representing 13-32% increase above the pre-dose levels. Moreover, heart rate (HR) increased by 38 ± 19 bpm from the baseline level (84 ± 12 bpm). HR peaked at 122 ± 30 bpm within 16 ± 9 min and returned to the baseline level after 113 ± 108 min. No significant changes in body temperature or blood oxygen saturations were observed. Individual data can be found in Appendix E.

Table 3-10. Mean ± SD changes in DBP (mmHg), SBP (mmHg), HR (bpm) after mephedrone administration

	DBP	**SBP**	**HR**
Baseline (mmHg or bpm)	77 ± 7	126 ± 13	84 ± 12
Peak (mmHg or bpm)	95 ± 9	155 ± 17	122 ± 30
Increased by (mmHg or bpm)	18 ± 5	29 ± 13	38 ± 19
Peak (min)	16 ± 15	7.5 ± 4.2	16 ± 9
Return to baseline (min)	93 ± 67	102 ± 54	113 ± 108

3.5.4 Discussion

Mephedrone produced increases in several VAS parameters related to stimulant-like effects and changes in perception. All subjective effects, except for “changes in light”, commenced and peaked after 15 min. VAS parameters returned to the pre-dose levels after 60-180 min, except for “good drug effect” which returned to baseline after 300 min. In the only other published study reporting changes in VAS parameters after oral mephedrone administration (200 mg), subjective effects commenced at 15 min,

peaked at approximately 45 min and were close to the pre-dose levels 2-3 h after administration [414]. Earlier peak in subjective effects observed in our study is linked to the route of administration being nasal insufflation. In contrast, subjective effects produced by a 100 mg oral dose of MDMA commenced slightly later (at approximately 45 min) and peaked between 1 h and 1.5 h. Subjective effects were experienced for 2-3 h and returned to the pre-dose levels 4 h after administration [414].

Mephedrone administration produced stimulant-like cardiovascular effects which were manifested by a mean increase from the baseline in HR (38 ± 19 bpm), SBP (29 ± 13 mmHg) and DBP (18 ± 5 mmHg) which occurred at 16 ± 9 min, 7.5 ± 4.2 min and 16 ± 15 min, respectively. DBP was the first to returned to baseline levels after 93 ± 67 min, followed by SBP after 102 ± 54 min and HR after 113 ± 108 min. In a previous study, where a higher dose of mephedrone (200 mg) was administered orally mephedrone produced greater mean increases from the baseline in HR (46 ± 31 bpm), SBP (54 ± 33 mmHg) and DBP (14 ± 17 mmHg) after approximately 45 min [414]. When 100 mg of mephedrone was given orally, HR increased by 10 ± 4 bpm, SBP by 9.7 ± 7.6 mmHg and DBP by 15 ± 5 mmHg from the baseline but the time-course of these effects was not reported [75]. Similarly to mephedrone, 100 mg of orally administered MDMA to 12 healthy volunteers resulted in mean increases in HR (19 ± 12 bpm) after 1.5 h as well as in SBP (33 ± 9 mmHg) and DBP (15 ± 5 mmHg) after 1 h [414]. HR, SBP and DBP returned to the pre-dose levels 6 h after administration.

Pharmacokinetic data did not reflect the pharmacodynamic observations very well. Mephedrone reached T_{max} in whole blood at approximately 55.0 ± 18.2 min and was close to undetectable levels on Day 2. Most subjected effects measured by VAS and the physiological effects commenced and peaked at approximately 15 min after drug administration, lasted 60-300 min and declined in parallel with mean whole blood mephedrone concentrations.

3.5.5 Conclusion

Following an intranasal administration of 100 mg of mephedrone hydrochloride, increases in heart rate, systolic blood pressure and diastolic blood pressure were observed. Moreover, mephedrone administration resulted in stimulant-like effects and changes in perception reported on the VAS scale. Subjective and physiological effects peaked earlier than the T_{max} reached by mephedrone in whole blood.

3.6 Overall conclusion

Analytical methods for quantification of mephedrone and five of its Phase I metabolites have been developed and successfully validated in whole blood, plasma and DBS. All analytes were detected in whole blood and plasma whereas only mephedrone, NOR and 4-CARBOXY were detected in the majority of DBS. Pharmacokinetic parameters were similar between whole blood and plasma, demonstrating rapid absorption of mephedrone following nasal insufflation. The mean whole blood to plasma drug distribution ratios suggest that mephedrone, DHM, HYDROXY and DHNM partition into the erythrocytes while NOR and 4-CARBOXY distribute into plasma. Even though a moderate to strong correlation was observed between plasma, DBS and whole blood concentrations, Bland-Altman analysis showed wide confidence intervals demonstrating poor agreement between methods. Moreover, enantiomers of mephedrone were successfully separated on a chiral column and quantified in whole blood for the first-time. The enantiomeric fraction calculated for samples collected between 30 min and 360 min has been shown to be statistically different from the expected value of 0.5, suggesting enantioselective pharmacokinetics. However, it is not yet clearly understood if the difference is clinically significant. Finally, mephedrone administration increased heart rate, systolic blood pressure, diastolic blood pressure and most of the visual analogue scale parameters.

CHAPTER 4

DETECTION OF MEPHEDRONE AND ITS METABOLITES IN FINGERPRINT SWEAT

4.1 Detection of mephedrone and its metabolites in fingerprint sweat

Non-invasive, easy and quick sample collection coupled with marked subject acceptability have recently made fingerprint sweat an attractive matrix for drug testing. A number of studies have previously reported the detection of illicit drugs (either parent, Phase I or Phase II metabolites) in sweat [157,349,350] but this is the first study investigating distribution of mephedrone and its metabolites in fingerprint sweat.

4.2 Fingerprint sweat aims

The primary aim of this study was to investigate the distribution of mephedrone and its metabolites in fingerprint sweat after a controlled administration. The secondary aim was to compare concentrations obtained in fingerprint sweat with those obtained in whole blood in order to assess correlation between the two matrices. Finally, the viability of fingerprint sweat for drug detection was evaluated.

4.3 Experimental

4.3.1 Reagents

Please refer to Section 3.1.3.1 in Chapter 3.

4.3.2 Blank matrix collection

'Blank' fingerprint sweat was collected from drug-free volunteers. Ethical approval for this collection of drug-free matrix was granted by the Research Ethics Committee at King's College London (HR 16/17 4237) and can be found in Appendix B. Fingertips were wiped with an ethanol wipe and allowed to dry. Glass cover slips (15 mm in diameter) were placed inside clean weighing boats and fingerprints were deposited on the surface of the cover slips.

4.3.3 Volunteer administration study and sample collection

Fingerprint sweat was collected from each finger (labelled as F1-F5, where F1 was the thumb and F5 was the little finger) of the right hand from 6 participants (referred to here as M1-M6) at -10 min (before administration), 10 min, 20 min, 45 min, 90 min, 3 h, 5 h, Day 2 and Day 3 after nasal insufflation of 100 mg of mephedrone. Before the -10 min and 10 min sample collection, fingertips were wiped with an ethanol wipe and allowed to dry. Fingertips were only cleaned at these two timepoints to remove external contamination at the -10 min sample collection and to wash off any residual mephedrone powder at 10 min after nasal insufflation. Natural sweat excretions were collected at all other timepoints. Fingerprint sweat was deposited on clean glass cover slips placed inside clean weighing boats. Glass cover slips were then transferred to 20 mL scintillation vials which were stored at -20°C until analysis. On several occasions glass cover slips broke under applied pressure. Broken pieces of the cover slips were also transferred to scintillation vials.

4.3.4 Calibration standards and quality control samples

Calibration standards were prepared in methanol (MeOH) at the following concentrations: mephedrone (MEPH), dihydro-nor-mephedrone (DHNM), nor-mephedrone (NOR) at 0.2, 1, 5, 10, 20, 40, 50 ng/mL; hydroxytolyl-mephedrone (HYDROXY) and 4-carboxy-mephedrone (4-CARBOXY) at 0.1, 1, 5, 10, 20, 40, 50 ng/mL;

and dihydro-mephedrone (DHM) at 0.16, 0.5, 1, 5, 10, 25, 50 ng/mL. Fingerprint sweat samples from drug-free users were deposited on the glass cover slips as described in 4.3.2. Two x 50 µL of each calibration standard was aliquoted on a cover slip and allowed to dry. QC Low (0.8 ng/mL for MEPH, DHNM, NOR; 0.5 ng/mL for HYDROXY and 4-CARBOXY; and 0.4 ng/mL for DHM), QC Med (10 ng/mL for MEPH, DHNM, NOR, HYDROXY, 4-CARBOXY; 5 ng/mL for DHM) and QC High (40 ng/mL for all analytes) were prepared in the same way as calibration standards. Internal standard (IS) containing MEPH-d_3 and DHM-d_3 at 250 ng/mL was prepared in MeOH.

Calibration standards and QCs were prepared fresh on the day of sample analysis. Blanks containing fingerprints but no IS and one sample containing a fingerprint and IS were also prepared and taken through the extraction.

4.3.5 Sample preparation

Fifty microliters of the IS was aliquoted on the glass cover slips and allowed to evaporate. Cover slips were then transferred into scintillation vials and 300 µL of 0.2% formic acid in ACN:Water (90:10 v/v) was added. Vials were sonicated for 7 min at 35 kHz and vortex mixed for 1 min at 1200 rpm. The extraction solvent was transferred to clean 1.5 mL Eppendorf tubes and evaporated at 45°C. Samples were reconstituted with 100 µL of 0.1% formic acid in ACN:Water (10:90 v/v).

Due to the nature of the collected samples, dilution could not be performed at the beginning of an extraction. As a result, 1 in 100 dilution was performed after sample reconstitution (see Section 2.6.7.7 in Chapter 2 for more details). Where dilution was required, 3 additional QCs were extracted and diluted in the same manner.

4.3.6 LC-MS/MS conditions

DHM-d_3 was used as an IS for 4-CARBOXY. For other details please refer to Section 3.1.3.7 in Chapter 3.

4.3.7 Pharmacokinetic calculations

Please refer to Section 2.3 in Chapter 2.

4.3.8 Validation procedures

Please refer to Section 2.5 in Chapter 2.

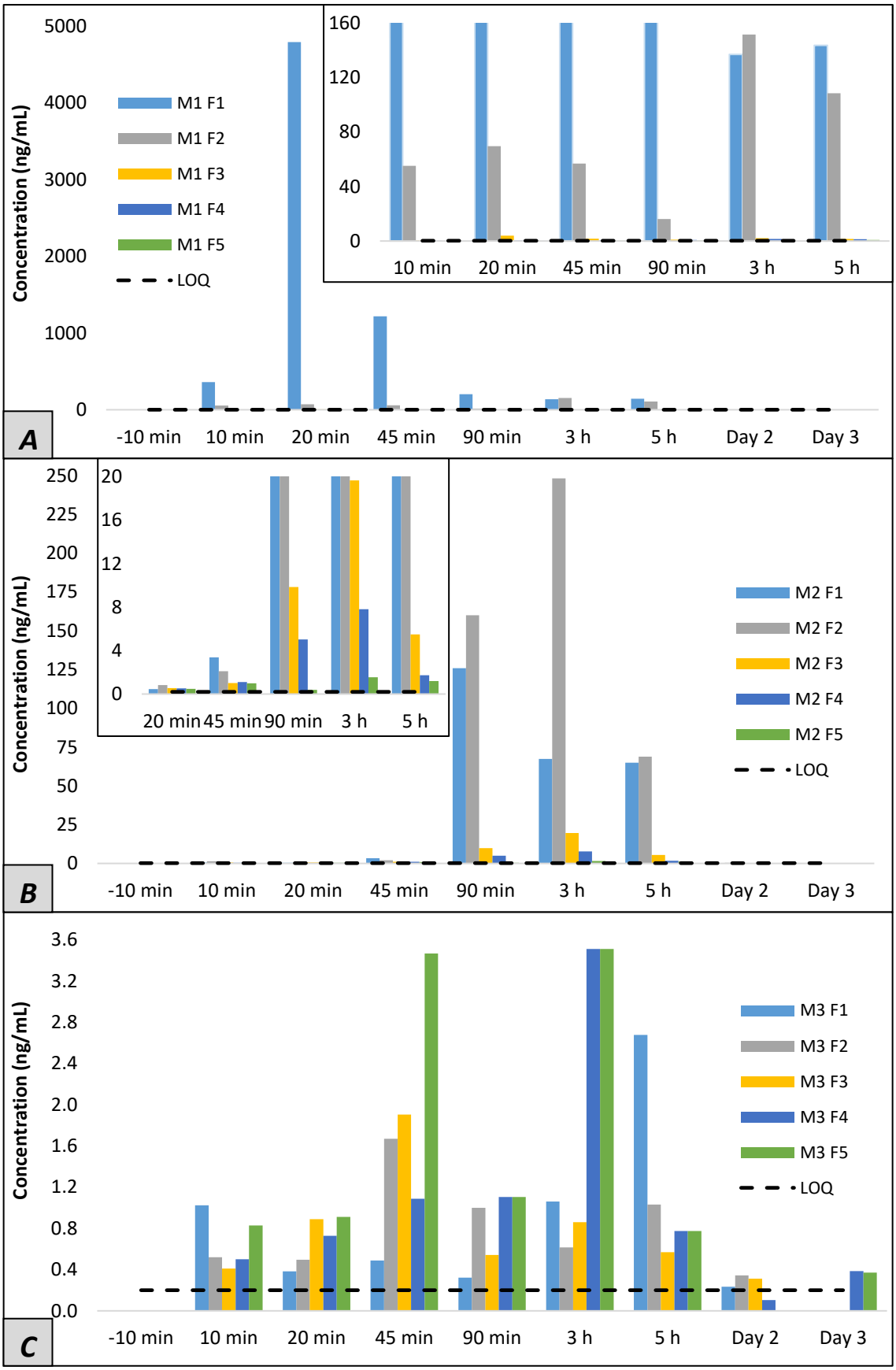
4.4 Results

4.4.1 Method validation

Please refer to Section 2.6.7 in Chapter 2.

4.4.2 Detection of mephedrone and its metabolites in fingerprint sweat

Mephedrone was detected in 163 (62%) of the 264 fingerprint sweat samples collected from 10 min until Day 3 and in the sweat from at least one finger in all participants (Figure 4-1). Mephedrone was first detected at 10 min in M1 in all fingers except F5, in M2 and M3 in all fingers, in M5 in all fingers except F2 and in M6 in F1 and F5 only. In M4 mephedrone was detected after 10 min in F3, after 90 min in F2 and F4 and after 3 h in F1. In F5 the analyte was only detected at 3 h. The last detected concentration was determined at the limit of detection (LOD) of 0.2 ng/mL and was observed between 5 h and Day 3 for M1-M6, except for F5 in M4 where it was detected at 3 h. Mephedrone was detected above the LOD in 11 (37%) fingerprint sweat samples collected on Day 2 and in 7 (23%) fingerprint sweat samples collected on Day 3. The analyte was present in 29 out of 30 (97%) fingerprint sweat samples collected at 5 h, the exception being F5 in M4.


A
Concentration (ng/mL)
5000
4000
3000
2000
1000
0
M1 F1
M1 F2
M1 F3
M1 F4
M1 F5
LOQ
-10 min
10 min
20 min
45 min
90 min
3 h
5 h
Day 2
Day 3
160
120
80
40
0
B
Concentration (ng/mL)
250
225
200
175
150
125
100
75
50
25
0
20
16
12
8
4
0
M2 F1
M2 F2
M2 F3
M2 F4
M2 F5
LOQ
C
Concentration (ng/mL)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
M3 F1
M3 F2
M3 F3
M3 F4
M3 F5
LOQ

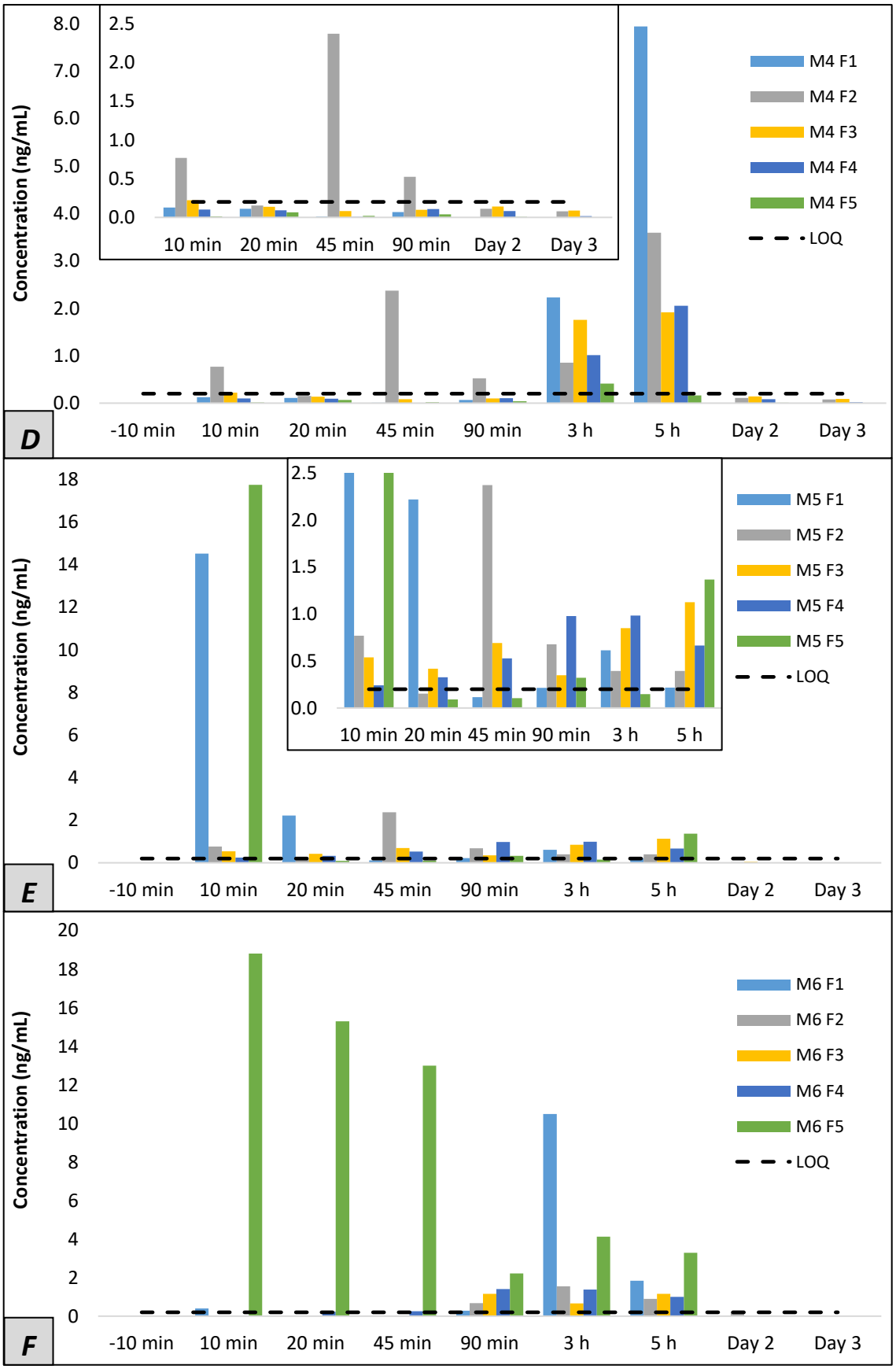


Figure 4-1. Concentration of mephedrone in A) M1, B) M2, C) M3, D) M4, E) M5, F) M6 in sweat collected from each finger (F1-F5); A), B), D) and E) also show zoomed in section of the graphs

NOR was detected in 7 (2.7%) fingerprint sweat samples above the limit of quantification (LOQ; 0.2 ng/mL) and in 10 (3.8%) fingerprint sweat samples above the LOD (0.05 ng/mL) in M1 and M2 only (Table 4-1). HYDROXY was only detected in M1 F1 at 20 min at 0.399 ng/mL (LOQ of 0.1 ng/mL). Other analytes were not detected.

Table 4-1. NOR concentrations (in ng/mL) detected above the LOQ of 0.2 ng/mL and LOD of 0.05 ng/mL (ticks) in fingerprint sweat (F1-F5) in M1 and M2

	M1					M2				
	F1	**F2**	**F3**	**F4**	**F5**	**F1**	**F2**	**F3**	**F4**	**F5**
-10 min	×	×	×	×	×	×	×	×	×	×
10 min	×	×	×	×	×	×	×	×	×	×
20 min	1.64	×	×	×	×	×	×	×	×	×
45 min	0.622	×	×	×	×	×	×	×	×	×
90 min	0.209	×	×	×	×	✓	0.214	×	×	×
3 h	×	×	×	×	×	✓	0.217	0.260	×	×
5 h	×	×	×	×	×	0.296	×	✓	×	×
Day 2	×	×	×	×	×	×	×	×	×	×
Day 3	×	×	×	×	×	×	×	×	×	×

4.4.3 Pharmacokinetic analysis

Pharmacokinetic parameters could not be accurately determined due to the significant inter- and intra-individual variation in detected concentrations of mephedrone (Figure 4-1). Therefore, it was decided to provide observed ranges for the C_{max} and T_{max}. In M1 C_{max} (range: 0.713-4792 ng/mL) was observed between 20 min and 5 h. In M2 C_{max} (range: 1.54-248 ng/mL) was observed at 3 h, except for F1 where the concentration peaked at 90 min. In M3 smaller variability in C_{max} (range: 1.10-3.51 ng/mL) was observed but T_{max} was between 45 min and 5 h. In M4, C_{max} (range: 0.411-1.92 ng/mL)

was reached at 5 h, except for F5 where T_{max} was observed at 3 h. In M5, C_{max} of 14.5 ng/mL and 17.7 ng/mL was observed after 10 min in F1 and F5, respectively. In the other fingerprints, C_{max} (range: 0.985-2.37 ng/mL) was observed between 45 min and 5 h. In M6, C_{max} (range: 1.15-18.8 ng/mL) was reached between 10 min and 5 h. The same C_{max} of 1.15 ng/mL was observed at 90 min and 5 h in F3 whereas C_{max} of 18.8 ng/mL was observed at 10 min in F5.

Pharmacokinetic parameters could not be determined from the NOR data because it was only quantified in 7 samples (across only 3 fingers and 2 participants).

4.4.4 Comparison with whole blood

Mephedrone detection window was longer in fingerprint sweat collected from M1, M2, M3 and M6 compared to whole blood whereby mephedrone was detected up to 6 h, except for M1 which was also detected on Day 2 (see Table 4-2). Moreover, mephedrone was detectable in sweat collected from at least one finger 72 h (Day 3) after drug administration in M1, M2 and M3.

Table 4-2. Comparison of mephedrone concentrations detected above the LOQ of 0.2 ng/mL in both whole blood and fingerprint sweat (F1-F5) on Day 2 and Day 3 in M1-M6

	M1		**M2**		**M3**	
	Blood	**Sweat**	**Blood**	**Sweat**	**Blood**	**Sweat**
Day 2	✓	F1-F4	×	F2, F4	×	F1-F3
Day 3	×	F1-F4	×	F1	×	F4, F5
	M4		**M5**		**M6**	
	Blood	**Sweat**	**Blood**	**Sweat**	**Blood**	**Sweat**
Day 2	×	×	×	×	×	F2
Day 3	×	×	×	×	×	×

Due to large intra- and inter-subject variability, correlation between concentrations found in whole blood and fingerprint sweat could not be reliably assessed.

4.5 Discussion

4.5.1 Inter-subject variability

A large inter-subject variability in mephedrone concentrations was observed, with concentrations in the same finger ranging from 4,792 ng/mL in M1 F1 to 2.68 ng/mL in M3 F1. There are several factors which could have contributed to the variability in this study. The difference in the pressure applied during fingerprint deposition, the angle and duration of contact with the glass cover slips were not controlled but have been shown to greatly vary between individuals or even between samples collected from the same individual on different occasions [428,429]. Furthermore, the 'amount' of collected fingerprint sweat was not measured during the collection process which might have led to inaccurate results. Several strategies could be employed to help overcome these limitations in future studies and in clinical or forensic practice. *Bailey et al.* controlled the pressure at which fingerprints were deposited by placing glass slides on a scale and making sure individuals applied a pressure of 400-1000 g [362]. An even more accurate method was developed by *Fieldhouse* who designed a device where a finger is placed on a slide and is pressed down with constant pressure from above for a required duration of time. The device produced reproducible and consistent results within and between participants and improved the quality of fingerprint deposits [428,430]. Moreover, compounds found in high abundance in sweat, such as creatinine or serine, could also be measured alongside analytes of interest as demonstrated by *Goucher et al* [350]. For example, concentration of creatinine in human sweat has been reported to be directly proportional to the concentration in plasma. Therefore, when a drug or its metabolite passes from whole blood to sweat, the ratio of drug to creatinine would be independent of the amount of sweat deposited on a glass cover slip. Furthermore, targeting endogenous compounds present in sweat could demonstrate that sweat was deposited on the collection device which could help explain situations like the one observed in this

study for M3 F5 where mephedrone concentration dropped at 90 min to 1.10 ng/mL and increased to 3.51 ng/mL at 3 h (Figure 4-1 C).

Interestingly, the highest mephedrone concentration between 10 min and 5 h was detected in the thumbs (F1) which might be linked to the 'amount' of sweat collected due to their big surface area and/or due to higher pressure applied [431]. Mephedrone concentrations were lower in the index fingers (F2) compared to thumbs but were significantly higher than those in the middle (F3), ring (F4) and little fingers (F5). This could be due to the index finger being a more dominant finger with more muscle strength.

Excessive sweating following drug use can also influence the volume of excreted sweat which may impact the results of sweat analysis. Mephedrone is a sympathomimetic drug which is expected to increase sweating. Sweating was reported in the first published case of analytically confirmed acute mephedrone toxicity [432] and in 5-10% of 150 acute mephedrone toxicity calls to the Swedish Poisons Information Centre [4,433]. However, it is not known what the overall effect of mephedrone or other sympathomimetic drugs is on the sweat gland function and how it impacts their concentration in fingerprint sweat.

4.5.2 Detected analytes

Mephedrone and NOR were found above the LOQ in 62% and 2.7% of all post amdinistration fingerprint sweat samples, respectively. The parent drug is expected to be found in the matrix but some of its more polar metabolites may not effectively incorporate into sweat. This has been observed in other studies where analysis of fingerprint sweat from drug users (methamphetamine) [434] or from a controlled administration (cocaine, codeine) [435] resulted in the parent drug being detected at high concentrations in the samples whereas the metabolite(s) were not detected or were detected at much lower concentrations. The evidential value of fingerprint sweat samples where metabolites are not detected is questionable because it does not exclude the possibility of external contamination, which is of particular concern in recreational

drug users handling mephedrone supplied as powder. However, more research looking at the effect of hand/finger washing after coming in contact with mephedrone is needed. In addition, it is suspected that a relatively low dose of administrated mephedrone contributed to low detectability of its metabolites in fingerprint sweat. Mephedrone users report taking 100-200 mg every hour or two hours, such that they use up to 1 g or more per session [30,68]. It is, therefore, likely that in high-dose clinical or forensic cases of mephedrone abuse this method would demonstrate the presence of both parent and one (or potentially more) of its metabolites.

To further increase analytical sensitivity fingerprint sweat from all fingers could be deposited onto the same cover slip, providing a cumulative measurement. Moreover, a more sensitive analytical approach, such as capillary-liquid chromatography coupled to nanospray mass spectrometry could be employed to quantify mephedrone metabolites in individual fingerprints.

4.5.3 Collection procedure

Collection of fingerprint sweat is an easy and quick process but some problems were encountered during the study. Glass cover slips are very thin and fragile. They broke on several occasions under excessive pressure applied during fingerprint deposition. Moreover, glass cover slips had to be transferred from weighing boats into scintillation vials which made them likely to be dropped in the process and might have also introduced a potential for contamination related to the clinicians handling cover slips after collection and then prior to the next collection. A more user-friendly method could involve collecting fingerprint sweat samples on bigger slides or on a drug screening cartridge developed by Intelligent Fingerprinting, if a point of care test is required [436]. Even though it has been shown that the deposition surface plays an important role in analyte stability and recovery, other surfaces (e.g. paper) could be explored. The use of triangular pieces of chromatography paper would allow a direct mass spectrometry analysis by paper spray [363], which has been explored in collaboration with the University of Surrey (data not shown here).

Even though participants nasally insufflated mephedrone in a separate room, wore gloves and washed their hands after drug administration, it is likely they used fingers to wipe their nose after nasally insufflating mephedrone, inadvertently picking up residual mephedrone powder which was not effectively removed by cleaning fingertips with an ethanol wipe. This might explain high concentration of mephedrone seen at 10 min or considerably higher concentrations of mephedrone in M1. Samples can also be prone to contamination during extraction, but analytes were not present in the blank samples extracted alongside fingerprint sweat samples.

Another problem with collecting fingerprint sweat is the inability to collect more than one sample at any time. This is because most (if not all) sweat would be deposited on the first cover slip leaving no (or very little) sample to be deposited on the subsequent cover slips. The lack of back-up samples presents a problem when a sample needs to be re-extracted, diluted or analysed as part of incurred sample reanalysis.

As mentioned before, the volume of excreted sweat was not controlled during sample collection. Moreover, the rate of sweat excretion is highly variable between individuals and depends on their diet, medication, age and psychological state [437]. *Sears et al.* have shown that a good quality fingerprint can be collected 120 min after washing hands with soap and water, suggesting a longer wait time between sample collections could be beneficial but not necessarily practical [437].

4.6 Conclusion

A relatively small dose of administered mephedrone coupled with analyte detection in individual fingerprints resulted in only mephedrone and nor-mephedrone being detected in the samples. Compared to whole blood, mephedrone was detectable in fingerprint sweat collected from 3 participants 72 h after drug administration. Large inter- and intra-subject variability was observed which can be attributed to the differences in pressure applied during fingerprint deposition, the angle and duration of contact with the deposition surface coupled with the inability to control the ‘amount’ of

collected sweat. Given these limitations fingerprint sweat may not be ideal for use in quantitative analysis until practical solutions to the problems discussed in this chapter are found.

CHAPTER 5

DETECTION OF MEPHEDRONE AND ITS METABOLITES IN URINE

5.1 Detection of mephedrone and its metabolites in urine

Due to the ease of collection and high drug concentrations, urine screens are one of the most common methods in drug testing. Only two controlled human administration studies have investigated the distribution of mephedrone and some of its metabolites in urine. In the first study 6 recreational drug users orally ingested 150 mg of mephedrone. Urine samples were collected between 0-4 h, 4-8 h, 8-12 h, 12-24 h and 24-48 h post drug administration and were analysed by LC-MS [91] and GC-MS [97]. The authors found 4-carboxy-mephedrone (4-CARBOXY) to be the most abundant analyte, reaching concentrations roughly 10 times higher than those of mephedrone (MEPH). Dihydro-mephedrone (DHM), nor-mephedrone (NOR) and N-succinyl nor-mephedrone were also detected but at lower concentrations. Mephedrone showed low urinary recovery, with only about 1.15% of total administered dose being recovered following LC-MS analysis [91] and 15.4 ± 2.6% following GC-MS analysis [97]. Renal clearance of 5.6 ± 2.6 L/h confirmed rapid elimination of mephedrone but was not reported for the metabolites. In another study, healthy subjects orally ingested different doses of mephedrone (n=3 took 50 mg and 100 mg; n=6 took 150 mg and 200 mg) [75]. Detected concentration were not reported as the publication focused on urinary recovery of the analytes following drug administration. The authors found that the urinary recovery of mephedrone and its metabolites was proportional to the administered doses [75].

5.2 Urine aims

The primary aim of this study was to quantify mephedrone and its metabolites in urine. The secondary aim was to investigate urinary recoveries and renal clearance which has not been reported before for mephedrone metabolites. The final aim was to compare the window of detection between urine and whole blood, looking specifically for metabolites which would be good markers of mephedrone use.

5.3 Experimental

5.3.1 Reagents

Please refer to Section 3.1.3.1 in Chapter 3.

5.3.2 Blank matrix collection

Urine was collected from drug-free volunteers into polyethylene Nalgene® bottles. Ethical approval for the collection of drug-free matrix was granted by the Research Ethics Committee at King's College London (HR 16/17 4237) and can be found in Appendix B.

5.3.3 Volunteer administration study and sample collection

Six healthy male volunteers nasally insufflated 100 mg of mephedrone hydrochloride. Urine samples were collected into polyethylene Nalgene® bottles at -10 min (0 h, before administration), 6 h, Day 2, Day 3 and Day 30. Urine samples were also collected between -10 min and 6 h if a participant felt the need to pass urine. The volume of excreted urine was recorded. Urine samples were stored at -20°C until analysis.

5.3.4 Working solutions

Working solutions used for the preparation of the calibration curve were prepared in MeOH:water (50:50 v/v) at 15, 50, 125, 250, 500, 1000, 1250 ng/mL for DHM, NOR, dihydro-nor-mephedrone (DHNM); and at 40, 100, 200, 250, 500, 1000, 1250 ng/mL for mephedrone, hydroxytolyl-mephedrone (HYDROXY), 4-CARBOXY. Working solution used for the preparation of the quality control (QC) samples at low, medium and high level were made in MeOH:water (50:50 v/v) at 25, 250, 1000 ng/mL for DHM, NOR, DHNM and at 50, 250, 1000 ng/mL for mephedrone, HYDROXY, 4-CARBOXY. Internal standard (IS) solution containing MEPH-d_3 and DHM-d_3 at 50 ng/mL was prepared in MeOH:water (50:50 v/v).

5.3.5 Calibration standards and quality control samples

Matrix-matched calibration standards containing DHM, NOR and DHNM at 0.6, 2, 5, 10, 20, 40, 50 ng/mL; and mephedrone, HYDROXY, 4-CARBOXY at 1.6, 4, 8, 10, 20, 40, 50 ng/mL were prepared by the addition of an appropriate volume of the working solution to urine. QC Low (1 ng/mL for DHM, NOR and DHNM; and 2 ng/mL for mephedrone, HYDROXY, 4-CARBOXY), QC Med (10 ng/mL for all analytes) and QC High (40 ng/mL for all analytes) were prepared by the addition of an appropriate volume of the working solution to urine.

Calibration standards and QCs were prepared fresh on the day of sample analysis. Blanks containing urine but no IS and one sample containing urine and IS were also prepared and taken through the extraction.

5.3.6 Sample preparation

Sample preparation described in Section 3.1.3.1 in Chapter 3 was followed but 250 µL of urine was taken through the extraction. Where dilution was required, samples were

diluted 1 in 100 or 1 in 1000 in the blank matrix alongside 3 additional QCs prepared in the same manner.

5.3.7 LC-MS/MS conditions

DHM-d_3 was used as an IS for 4-CARBOXY. For other details please refer to Section 3.1.3.7 in Chapter 3.

5.3.8 Pharmacokinetic calculations

Please refer to Section 2.3 in Chapter 2.

5.3.9 Validation procedure

Please refer to Section 2.5 in Chapter 2.

5.4 Results

5.4.1 Method validation

Please refer to Section 2.6.4 in Chapter 2.

5.4.2 Concentrations of mephedrone and its metabolites in urine

Concentrations of mephedrone and its metabolites in urine collected from 6 participants (M1-M6) are shown in Figure 5-1 - Figure 5-6. Where data for Day 2, Day 3 or Day 30 is not shown, analytes were not detected at these timepoints. A urine sample was not provided in the first 2 h post administration by M6 and so there is no data available for that time period. Analytes were not detected in the pre-administration (-10 min) urine samples (data not shown).

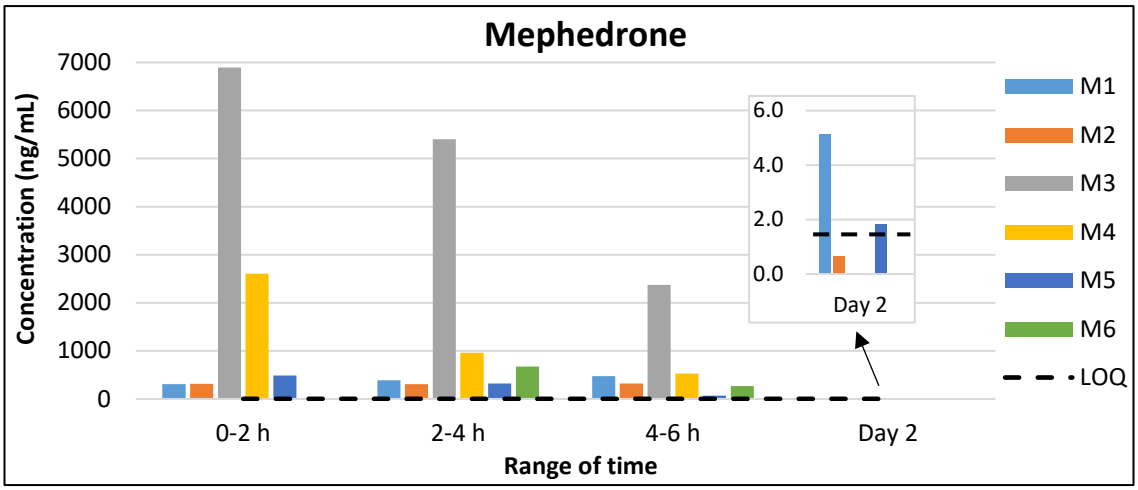


Figure 5-1. Concentration of mephedrone in urine collected from M1-M6

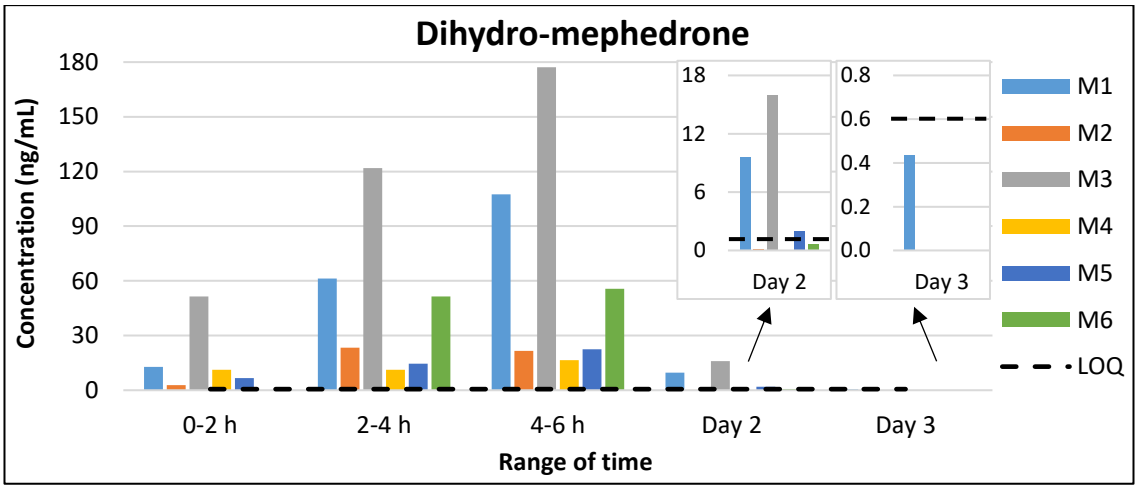


Figure 5-2. Concentration of dihydro-mephedrone in urine collected from M1-M6

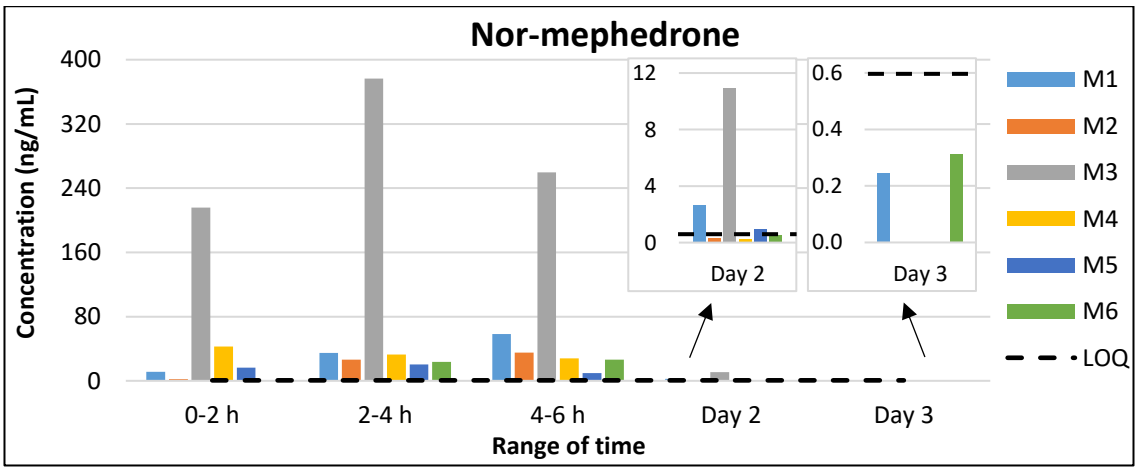


Figure 5-3. Concentration of nor-mephedrone in urine collected from M1-M6

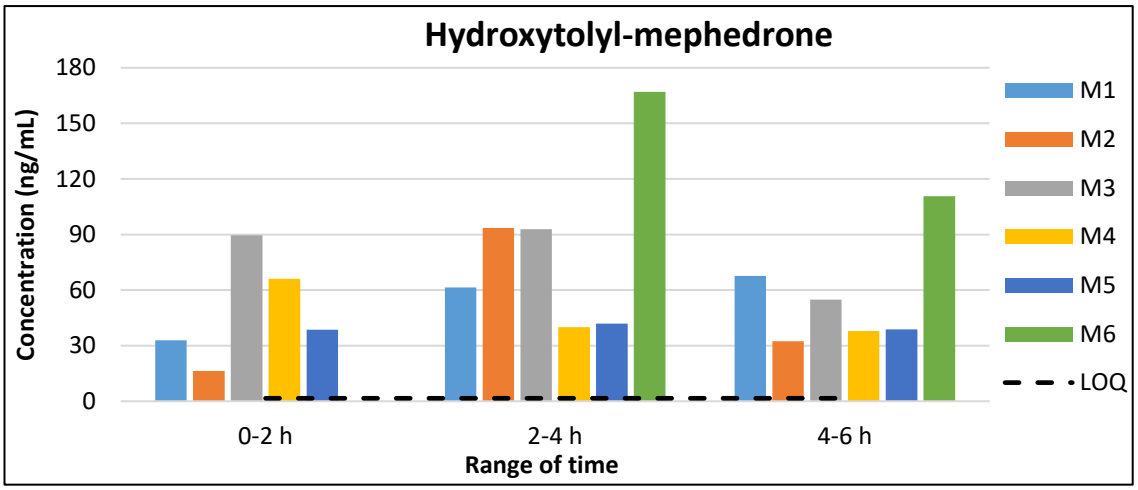


Figure 5-4. Concentration of hydroxytolyl-mephedrone in urine collected from M1-M6

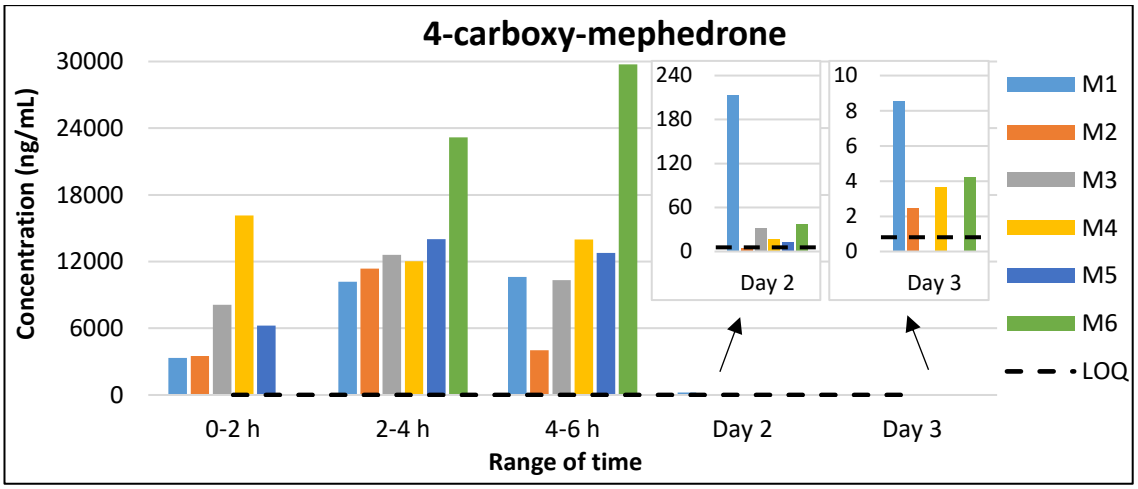


Figure 5-5. Concentration of 4-carboxy-mephedrone in urine collected from M1-M6

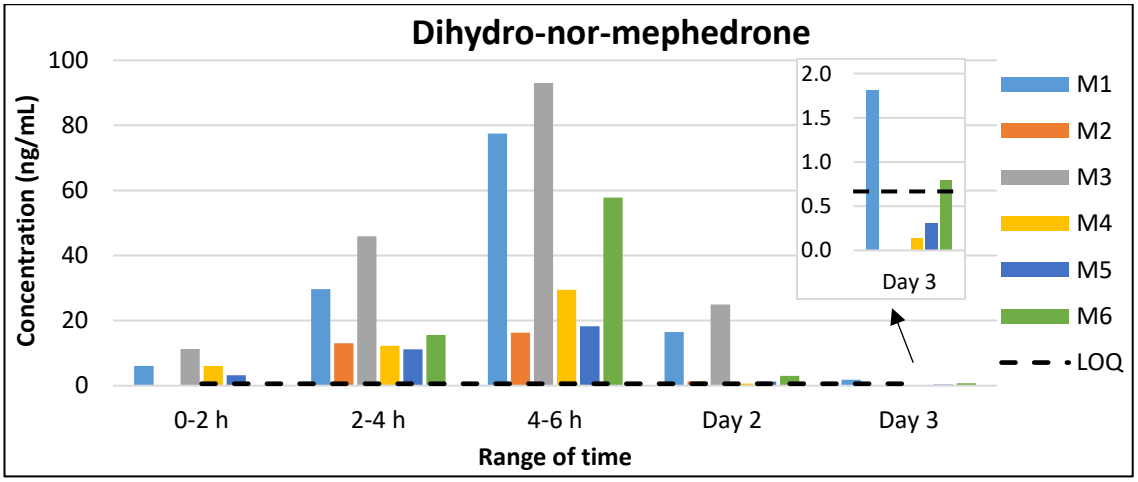


Figure 5-6. Concentration of dihydro-nor-mephedrone in urine collected from M1-M6

4-CARBOXY reached the highest concentrations in urine (range: 2.45-29,757 ng/mL), followed by mephedrone (range: 1.83-6,898 ng/mL) and NOR (range: 0.955-377 ng/mL). DHM (range: 0.675-177 ng/mL) and HYDROXY (range: 16.2-167 ng/mL) were detected at lower concentrations. DHNM was found at the lowest concentration, ranging from 0.600 ng/mL to 93.1 ng/mL. Mephedrone concentration peaked within 2 h and then started to decline as the metabolite concentrations started to rise. The highest concentration of mephedrone, DHM, NOR and DHNM between 0-6 h were detected in M3, which showed considerably higher mephedrone and NOR concentrations compared to other participants.

Analytes exhibited a wide range of detection windows in urine. Mephedrone was present in three participants (M1, M2, M5) on Day 2 but was undetectable on Day 3. DHM was detected in four participants (M1, M3, M5, M6) on Day 2 but only in M1 on Day 3. NOR, 4-CARBOXY and DHNM were all detectable in all participants on Day 2, with 4-CARBOXY and DHNM also being detectable in participants on Day 3. HYDROXY was detectable up to 6 h but declined to undetectable levels on Day 2 in all participants. No analytes were detected on Day 30.

5.4.3 Comparison with whole blood

Table 5-1 compares analytes detected above the LOD and LOQ on Day 2 in whole blood and urine. In whole blood, mephedrone was the only analyte detected after 6 h but only in M1 on Day 2. On Day 2 in urine mephedrone was detectable in M1, M2 and M5 whereas DHM was found in all participants except M4. NOR, 4-CARBOXY and DHNM were all detected above the LOD in all participants on Day 2.

Table 5-1. Comparison of analytes detected above the LOD (ticks) and LOQ (in ng/mL) in whole blood and urine samples collected on Day 2; ND – not detected

Participant	Matrix	MEPH	DHM	NOR	HYDROXY	4-CARBOXY	DHNM
M1	**Urine**	5.13	9.56	2.64	ND	213	16.5
	Blood	0.212	ND	ND	ND	ND	ND
M2	**Urine**	✓	✓	✓	ND	4.00	1.29
	Blood	ND	ND	ND	ND	ND	ND
M3	**Urine**	ND	16.0	10.9	ND	32.6	24.9
	Blood	ND	ND	ND	ND	ND	ND
M4	**Urine**	ND	ND	✓	ND	16.8	0.600
	Blood	ND	ND	ND	ND	ND	ND
M5	**Urine**	1.83	1.98	0.955	ND	12.9	1.22
	Blood	ND	ND	ND	ND	ND	ND
M6	**Urine**	ND	0.675	0.525	ND	37.6	3.00
	Blood	ND	ND	ND	ND	ND	ND

5.4.4 Urinary recovery

The minimum, maximum and mean urinary recovery of mephedrone relative to the administered dose of 100 mg in the first 6 h post administration is shown in Table 5-2. Only 1.36 ± 1.75% of unchanged mephedrone was recovered in urine. The highest urinary recovery of 4.85% (27.4 µmol) was seen in M3 while the lowest urinary recovery of 0.153% (0.864 µmol) was reported for M6. Standard deviation (SD) presented in Table 5-2 was larger than the mean due to exceptionally high mephedrone concentrations in M3.

Table 5-2. Urinary recovery of mephedrone relative to the administered dose of 100 mg

Participant	Mephedrone urinary recovery (%)	Mephedrone urinary recovery (µmol)
M1	0.759	4.28
M2	0.604	3.41
M3	4.85	27.4
M4	1.21	6.85
M5	0.549	3.10
M6	0.153	0.864
Mean	1.36	7.65
SD	1.75	9.86
Minimum recovery	0.153	0.864
Maximum recovery	4.85	27.4

Urinary recovery of mephedrone metabolites expressed as a percentage of the total dose and as a percentage of recovered mephedrone is presented in Table 5-3. DHM, NOR, HYDROXY and DHNM excretions were 3.90 ± 3.99%, 5.90 ± 8.46%, 5.26 ± 1.81% and 2.28 ± 1.76% of the total mephedrone eliminated in urine, respectively. 4-CARBOXY was found at much higher concentration in urine, representing 904 ± 162% of the recovered mephedrone.

Table 5-3. Minimum, maximum and mean urinary recoveries (% of the total dose) for mephedrone metabolites calculated from the time of drug administration up to 6 h (n=6)

Analyte	Mean recovery (%) ± SD	Minimum recovery (%)	Maximum recovery (%)	% of recovered MEPH ± SD
DHM	0.053 ± 0.054	0.012	0.149	3.90 ± 3.99
NOR	0.080 ± 0.115	0.008	0.313	5.90 ± 8.46
HYDROXY	0.072 ± 0.025	0.044	0.099	5.26 ± 1.81
4-CARBOXY	12.3 ± 2.20	8.77	15.4	904 ± 162
DHNM	0.031 ± 0.024	0.012	0.069	2.28 ± 1.76

5.4.5 Renal clearance

Renal clearance is defined as the volume of plasma cleared of a drug by kidneys per unit of time. Renal clearance, presented in Table 5-4, was calculated for all analytes and participants for the first 6 h post administration. HYDROXY displayed the greatest renal clearance (507 ± 226 mL/min), followed by 4-CARBOXY (349 ± 133 mL/min) and DHNM (252 ± 294 mL/min). NOR had the lowest renal clearance of 53.6 ± 67.0 mL/min. Renal clearance could not be determined for DHNM in M4 and M5 because the analyte was not detected in plasma samples in these participants. Renal clearance calculated for all analytes in M6 was considerably smaller compared to other participants.

Table 5-4. Summary of renal clearance (mL/min) calculated for all analytes and participants based on the data collected up to 6 h post mephedrone administration

Participant	MEPH	DHM	NOR	HYDROXY	4-CARBOXY	DHNM
M1	55.9	294	35.2	701	423	16.6
M2	41.1	134	24.0	443	359	320
M3	383	378	187	601	435	640
M4	117	125	45.3	671	335	-
M5	43.6	94.2	27.8	540	448	-
M6	5.55	27.2	2.22	87.2	92.3	31.3
Mean	108	175	53.6	507	349	252
SD	140	132	67.0	226	133	294
Minimum clearance	5.55	27.2	2.22	87.2	92.3	16.6
Maximum clearance	383	378	187	701	448	640

5.4.6 Renal clearance compared to the total body clearance

Drugs are removed from the blood by different mechanisms, pathways and organs. Liver and kidneys are usually thought to be the main clearing organs, but some drugs can be cleared by the lungs, bile or other organs/pathways. Therefore, renal clearance is only one component which constitutes total body clearance.

Table 5-5 compares renal clearance with total body clearance reported for plasma in Chapter 3 (Section 0). Renal clearance was lower for all analytes compared to the total body clearance.

Table 5-5. Comparison of renal clearance (mL min^{-1} kg^{-1}) calculated for all analytes and participants based on the data collected for up to 6 h post mephedrone administration with the total body clearance (mL min^{-1} kg^{-1})

Participant	MEPH	DHM	NOR	HYDROXY	4-CARBOXY	DHNM
M1	0.67	3.49	0.42	8.35	5.04	0.20
M2	0.54	1.75	0.31	5.80	4.70	4.19
M3	5.78	5.70	2.82	9.07	6.57	9.66
M4	1.98	2.11	0.77	11.4	5.68	-
M5	0.53	1.15	0.34	6.57	5.44	-
M6	0.10	0.49	0.04	1.58	1.67	0.57
Mean	1.6	2.4	0.8	7.1	4.9	3.7
SD	2.1	1.9	1.0	3.4	1.7	4.4
Total body clearance	66.5 ± 23.6	2678 ± 2357	468 ± 332	9161 ± 8595	23.7 ± 7.87	-

5.5 Discussion

4-CARBOXY was the most abundant metabolite in urine, followed by NOR and HYDROXY. In a controlled administration study performed by *Olesti et al.* 4-CARBOXY was also the major metabolite detected in urine reaching concentrations roughly 10 times higher than mephedrone [91]. Inter-subject variability was observed, with M3 showing the highest concentrations of mephedrone, DHM, NOR and DHNM between 0-6 h. This could be due to the polymorphic nature of CYP2D6 which is responsible for mephedrone metabolism [70], but other factors not yet defined could also be at play.

Polar Phase II metabolites conjugated with charged species, such as glutathione, sulfate, glycine or glucuronic acid, are readily excreted in urine. Conjugated metabolites are often cleaved during toxicological analysis via hydrolysis (performed enzymatically with β-glucuronidase or chemically with hydrochloric acid or sodium hydroxide), which increases concentrations of Phase I metabolites in urine. In this study, hydrolysis was not carried out nor were the intact Phase II metabolites targeted. In other studies, Phase II metabolites of mephedrone conjugated with glucuronic acid were detected in rat urine [73], in rat liver hepatocytes [71] and in human urine [74]. Interestingly, mephedrone conjugation with succinic acid has also been reported in human urine [74] whereas following the analysis of rat urine NOR has been shown to conjugate with succinic, glutaric and adipic acid [73].

According to a review describing cases of clinical mephedrone intoxication, mean mephedrone urinary concentration was 50,476 ng/mL, ranging from 1 ng/mL to 198,000 ng/mL [407]. However, in many of these cases the exact mephedrone dose was unknown and the route of administration varied between cases. To my knowledge only one study has reported the presence of mephedrone metabolites in a urine sample collected from a forensic traffic case in Denmark. DHM, NOR, HYDROXY and 4-CARBOXY were detected but concentrations were not reported, except for HYDROXY which was found at 40 μg/kg [70]. In our study, mephedrone concentrations were between

1.83 ng/mL and 6,898 ng/mL (mean: 1,001 ng/mL). In other controlled human administration studies, mephedrone urinary concentrations over a 4 h period were 298 ng/mL (after a 50 mg oral dose), 845 ng/mL (after a 100 mg oral dose) and 2,824 ng/mL (after a 150 mg oral dose) [407].

Compared to whole blood, where mephedrone was detected in one sample collected on Day 2, urine offered a longer detection window. 4-CARBOXY and DHNM were the only metabolites detectable in the majority of urine samples on Day 3, making them promising markers of mephedrone use.

From the total administered dose of 100 mg only about 1.36% (1.36 mg or 7.65 µmol) of mephedrone was recovered in urine in a 6 h period. That is in close agreement with a previously reported total urinary recovery of about 1.15% (9.45 ± 2.92 µmol) calculated from urine samples collected continuously for 48 h following an oral administration of 150 mg of mephedrone hydrochloride [91]. In our study, urine samples were not collected between the 6 h timepoint and Day 2, where mephedrone was still likely excreted, and so the 1.36% urinary recovery does not represent total urinary recovery.

Mephedrone has been shown to be rapidly eliminated with mean renal clearance of 108 ± 140 mL/min (6.48 ± 8.40 L/h). Previously reported renal clearance of 5.6 ± 2.6 L/h for mephedrone was based on urine samples collected over a 48 h period following an oral administration of 150 mg of mephedrone hydrochloride [97]. Moreover, renal clearance of mephedrone metabolites has been reported for the first-time in our study. The two most polar metabolites (HYDROXY and 4-CARBOXY) had the largest renal clearance, which has likely resulted in HYDROXY not being detectable in urine after 6 h. DHNM had a moderate renal clearance of 252 ± 294 mL/min and was still detectable in urine on Day 2 and in urine samples from four participants on Day 3.

As expected, renal clearance was lower than the total body clearance. However, it needs to be noted that renal clearance was calculated from the data collected up to 6 h whereas total body clearance was calculated from the data collected up to Day 3 post mephedrone administration. Moreover, urine samples were not subjected to hydrolysis which would have cleaved the analytes from their intact Phase II conjugates nor were the Phase II metabolites targeted. Consequently, conjugated analytes are not accounted for in the results presented here, likely underestimating renal clearance.

5.6 Conclusion

Following nasal insufflation of mephedrone all analytes were detected in urine, where 4-CARBOXY was the most abundant. 4-CARBOXY and DHNM were the only metabolites detectable in urine samples on Day 3, making them promising markers of mephedrone use. This is a crucial finding for the clinical and forensic toxicologists who by targeting 4-CARBOXY and DHNM in urine may be able to prove mephedrone use.

CHAPTER 6

DETECTION OF MEPHEDRONE AND ITS METABOLITES IN HEAD HAIR AND ORAL FLUID

6.1 Head hair

6.1.1 Detection of mephedrone and its metabolites in head hair

Head hair is a unique matrix which allows retrospective detection of drug use. However, results of head hair analysis can be difficult to interpret due to many factors (e.g. hair colour, hair treatment, medication, age) impacting the degree of drug incorporation into hair [173,216]. Moreover, environmental contamination, which can often lead to false-positive results, is a significant pitfall of hair testing [217]. Currently, the Society of Hair Testing (SoHT) recommends the following four approaches to differentiate drug administration from external contamination: the use of cut-off levels, targeting metabolites, analysis of washes collected during hair decontamination and calculation of metabolite to parent drug concentration ratios [215]. The cut-off concentrations for both screening and confirmatory analysis for the well-established drugs (e.g. cocaine and amphetamines) have been proposed by SoHT [215] and by the Substance Abuse and Mental Health Services Administration [177], but they do not exist for new psychoactive substances, such as mephedrone.

Mephedrone (MEPH) has been targeted in hair samples collected from suspected drug users and analysed as part of forensic casework [116,377–381]. Quantitative results were only presented in some of these studies. Following LC-MS analysis, *Salomone et al.* detected mephedrone in two samples from proven MDMA and/or ketamine users at 50 pg/mg and 59 pg/mg [380]. *Martin et al.* found mephedrone at higher concentrations of

26.8 ng/mg (range: 0.2-313.2 ng/mg) in hair samples submitted to the ChemTox Laboratory in France [381]. Post-mortem hair concentrations of mephedrone were reported by *Torrance et al.* who detected the drug at concentrations between 4.2 ng/mg and 4.7 ng/mg following GC-MS analysis [116]. The only method which targeted mephedrone metabolites (nor-mephedrone and dihydro-nor-mephedrone) by LC-MS did not detect them in the hair samples from 154 healthy volunteers [378].

6.1.2 Head hair aims

The primary aim of this study was to determine the concentration of mephedrone and its metabolites in head hair. The second aim was to calculate the concentration ratios between metabolites and mephedrone, which have not been reported before.

6.1.3 Experimental

6.1.3.1 Reagents

In addition to the reagents described in Chapter 3 (Section 3.1.3.1), sodium hydroxide was purchased from VWR (Lutterworth, UK). The pulveriser (Pulverisette 23) and metal beads used for pulverising hair were purchased from Fritsch (Brackley, UK).

6.1.3.2 Blank matrix collection

Hair samples (approximately 7 mm thick) were collected from drug-free volunteers. Samples were collected from the posterior vertex by cutting as close to the scalp as possible. The root end was clearly marked and stored in a paper envelope kept in the dark and at room temperature. Ethical approval for the collection of drug-free matrix was granted by the Research Ethics Committee at King's College London (HR 16/17 4237) and can be found in Appendix B.

6.1.3.3 Preparation of the blank matrix

Hair samples were washed with acetone (3 x 2 mL) and were left to dry overnight. Washes were collected individually, dried down, reconstituted in 100 µL of 0.1% formic acid in ACN:water (10:90 v/v) and analysed as described in 6.1.3.8. Washed hair samples were cut into 1 cm long segments using a scalpel blade and were pulverised at 35 Hz for 10 min or until fine powder was obtained. Ten ± 1 mg of pulverised hair was weighed out and transferred into glass tubes. Pulverised hair was then spiked with an appropriate solution as described in 6.1.3.6. The pulveriser, which was used to increase sample homogeneity, was thoroughly cleaned with methanol and water after every use.

6.1.3.4 Volunteer administration study and sample collection

Six healthy male volunteers nasally insufflated 100 mg of mephedrone hydrochloride. Head hair samples were collected at two timepoints as described in 3.1.3.2: -10 min (before administration) and Day 30. For the samples collected at -10 min, 0-1 cm hair segments were analysed whereas for the Day 30 samples, 0-1 cm and 1-2 cm hair segments were analysed (see 6.1.3.7 for sample preparation details).

The initial ethics application did not mention sample collection at -10 min and so these samples were not collected from the first 3 participants (M1-M3). The ethics application was later amended to allow the -10 min sample collection from the last 3 participants (M4-M6). Hair colour and type is presented in Table 6-1 (note that participant M4 had their hair dyed purple at the time of enrolment to the study).

Table 6-1. Hair type and colour of the six participants who took part in the study

Participant	Hair type	Hair colour
M1	Straight	Brown
M2	Straight	Brown
M3	Straight	Black
M4	Straight	Black/Purple (dyed)
M5	Curly	Brown
M6	Straight	Brown

6.1.3.5 Working solutions

Working solutions used for the preparation of the calibration curve were prepared in methanol (MeOH) at 2, 6, 8, 10, 12, 15, 20 ng/mL for MEPH; 0.4, 0.8, 1.5, 2, 8, 15, 20 ng/mL for dihydro-nor-mephedrone (DHNM); 0.4, 1, 2, 4, 15, 25, 40 ng/mL for nor-mephedrone (NOR); 1, 2.5, 5, 6, 10, 20, 40 ng/mL for hydroxytolyl-mephedrone (HYDROXY); 4, 10, 15, 20, 25, 35, 40 ng/mL for 4-CARBOXY and DHM. Working solution used for the preparation of the quality control (QC) samples at QC Low (5 ng/mL for MEPH; 0.5 ng/mL for DHNM; 0.8 ng/mL for NOR; 2 ng/mL for HYDROXY; and 8 ng/mL for 4-CARBOXY and DHM), QC Med (10 ng/mL for MEPH; 2 ng/mL for DHNM; 4 for NOR; 6 ng/mL for HYDROXY; and 19 ng/mL for 4-CARBOXY and DHM) and QC High (17 ng/mL for MEPH; 16 ng/mL for DHNM; 33 ng/mL for NOR; 28 ng/mL for HYDROXY; and 34 ng/mL for 4-CARBOXY and DHM) were prepared in MeOH.

6.1.3.6 Calibration standards and quality control samples

Matrix-matched calibration standards containing MEPH at 20, 60, 80, 100, 120, 150, 200 pg/mg; DHM and 4-CARBOXY at 40, 100, 150, 200, 250, 350, 400 pg/mg; NOR at 4, 10, 20, 40, 150, 250, 400 pg/mg; HYDROXY at 4, 8, 15, 20, 80, 150, 200 pg/mg; and DHNM at 4, 8, 15, 20, 80, 150, 200 pg/mg were prepared by aliquoting 100 µL of an appropriate working solution to pulverised hair (10 ± 1 mg). QC Low (50 pg/mg for MEPH; 80 pg/mg

for DHM and 4-CARBOXY; 8 pg/mg for NOR; 20 pg/mg for HYDROXY; 5 pg/mg for DHNM), QC Med (100 pg/mg for MEPH; 190 pg/mg for DHM and 4-CARBOXY; 8 pg/mg for NOR; 20 pg/mg for HYDROXY; 5 pg/mg for DHNM) and QC High (170 pg/mg for MEPH; 340 pg/mg for DHM and 4-CARBOXY; 330 pg/mg for NOR; 280 pg/mg for HYDROXY; 160 pg/mg for DHNM) were prepared in the same way as calibration standards. Internal standard (IS) solution containing MEPH-d_3 and DHM-d_3 was prepared at 50 ng/mL in MeOH.

Calibration standards and QCs were prepared fresh on the day of sample analysis. Blanks containing pulverised hair but no IS and one sample containing pulverised hair and IS were also prepared and taken through the extraction.

6.1.3.7 Sample preparation

Hair samples collected from the participants were washed, pulverised and weighed as described in 6.1.3.3. Following the addition of 50 µL of the IS into glass tubes with pulverised hair, 1 mL of 0.1 M sodium hydroxide$_{(aq)}$ was added. Tubes were capped, and samples were incubated at room temperature for 30 min with shaking. Following centrifugation at 2300 rpm for 5 min, samples were extracted by solid phase extraction (SPE) as described in Chapter 3 (Section 3.1.3.6). Samples did not require dilution.

6.1.3.8 LC-MS/MS conditions

DHM-d_3 was used as an IS for 4-CARBOXY. For other details please refer to Section 3.1.3.7 in Chapter 3.

6.1.3.9 Validation procedure

Please refer to Section 2.5 in Chapter 2.

6.1.4 Results

6.1.4.1 Method validation

Please refer to Section 2.6.8 in Chapter 2.

6.1.4.2 Concentrations of mephedrone and metabolites in head hair

As shown in Figure 6-1, mephedrone was detected in 0-1 cm hair segments collected on Day 30 from M1-M6. Mephedrone was quantified in M3 (48 pg/mg) and M6 (22 pg/mg) on Day 30 in the 0-1 cm segment and it was also detected below the limit of quantification (LOQ; 20 pg/mg) in M1, M4 and M5. In addition, mephedrone was found above the limit of detection (LOD; 5 pg/mg) on Day 30 in the 1-2 cm hair segment in M5. The concentrations shown below the LOQ in Figure 6-1 and Figure 6-2 cannot be reliably quantified and so these values are only indicative.

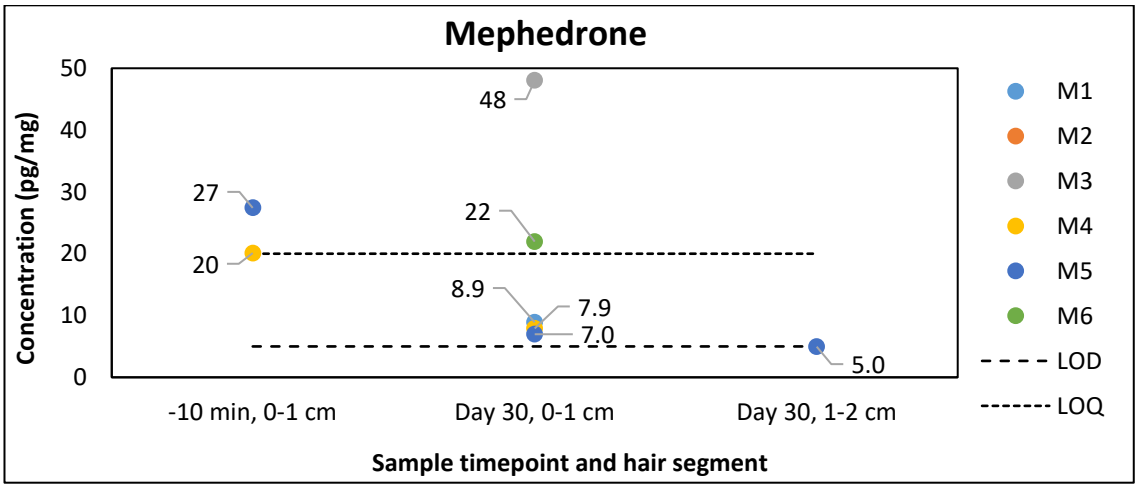


Figure 6-1. Mephedrone concentrations in head hair samples (1 cm segments) collected before mephedrone administration (M4-M6) and on Day 30 (M1-M6)

As shown in Figure 6-2, NOR was not detected in the 1-2 cm segment on Day 30 but was present above the LOQ (4 pg/mg) in the 0-1 cm segment in M3 at 9.10 pg/mg on Day 30. In addition, NOR was found above the LOD (1 pg/mg) on Day 30 in the 0-1 cm hair segment in M4-M6.

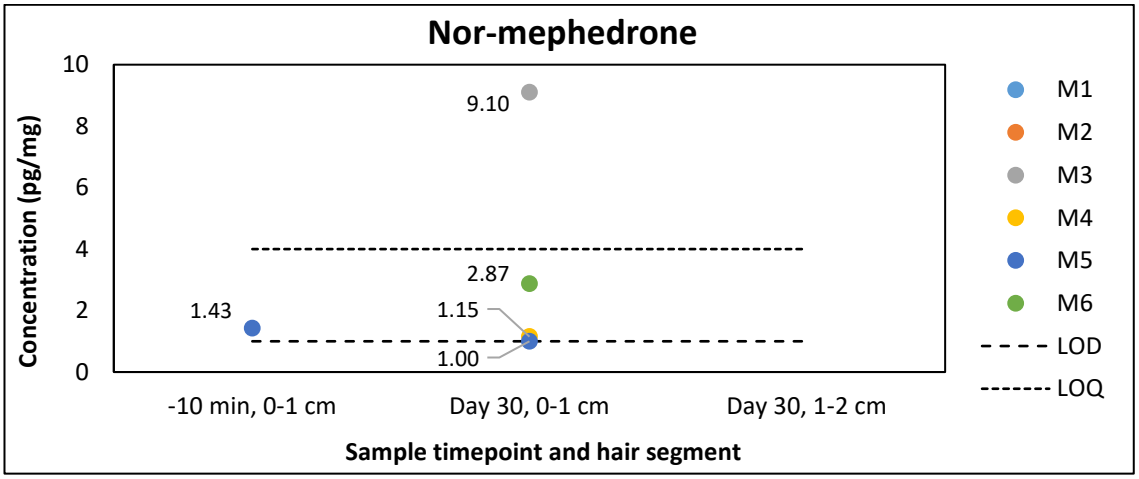


Figure 6-2. Nor-mephedrone concentrations in head hair samples (1 cm segments) collected before mephedrone administration (M4-M6) and on Day 30 (M1-M6)

Interestingly, mephedrone and NOR were also detected in the 0-1 cm hair segment from M4 and M5 collected before mephedrone administration (-10 min). Surface contamination of the hair was not detected in the acetone washes.

Other mephedrone metabolites were not detected, except for DHM which was found below the LOQ (40 pg/mg) and DHNM detected at 10.1 pg/mg in M3 in the 0-1 cm hair segment collected on Day 30.

6.1.4.3 Metabolite to parent drug concentration ratios

Metabolite to parent drug concentration ratios could only be calculated for one sample (M3 Day 30, 0-1 cm segment) where mephedrone and a metabolite were detected

above the LOQ. Calculated NOR:MEPH and DHNM:MEPH ratios were 0.19 and 0.21, respectively.

6.1.5 Discussion

The two polar metabolites (HYDROXY and 4-CARBOXY) were not detected in head hair. Even though polar metabolites are less likely to get incorporated into hair, there are reported cases of water-soluble metabolites, such as ecgonine methyl ester (cocaine metabolite), being found in hair samples obtained from the National Institute of Toxicology and Forensic Sciences [438] and from a controlled intranasal (100 mg) cocaine administration [439]. Even when polar metabolites get incorporated into hair, they may be easily washed off during regular haircare routine. In this study, poor recovery of 4-CARBOXY (4.55 ± 1.82% at QC Low and 5.58 ± 8.90% at QC High) might have also contributed to the absence of this analyte in head hair.

Interestingly, -10 min samples collected from M4 and M5 before mephedrone administration were positive for mephedrone. In addition, NOR was detected in the -10 min sample collected from M5. Participants in the study were occasional recreational drug users, including mephedrone. The study protocol asked participants to abstain from taking drugs 2 weeks before the administration day. The abstinence was confirmed by the analysis of a urine sample collected 7 days before the administration day, which was negative in all cases. M4 came to the screening visit on the 25th April and took mephedrone on the 11th June whereas M5 was screened on the 1st May and attended the administration day on the 18th June. In both cases the time period between the screening visit and the administration day was over a month. This likely created an opportunity for the participants to take drugs, including mephedrone, which would not have been against the study protocol if it had happened earlier than 2 weeks before the administration day. However, participants were not asked about the drug use between the screening visit and the administration day. Samples can also become contaminated during sample extraction, but this is highly unlikely as analytes were not detected in the blanks run directly before the -10 min samples.

The mechanism of drug incorporation into hair is not yet fully understood. Active or passive diffusion from the bloodstream or diffusion from sweat have been suggested [174]. The diffusion of analytes from sweat, sebum or other excretions onto the scalp is especially problematic because it leads to the growing or mature hair shafts bathing in these secretions. *Xiang et al.* monitored the diffusion of ketamine and norketamine from sweat and sebum by swabbing the posterior vertex after administration of a 10 mg ketamine dose [440]. All cotton swabs were positive for both analytes which demonstrated that the head hair was contaminated by sweat and sebum. In this study the posterior vertex was not swabbed and so the impact of skin excretions on analyte concentrations in hair cannot be established but it should be investigated in future studies.

Natural hair colour has been shown to play an important role in drug incorporation into hair. Drugs of abuse bind to melanin which is present as either eumelanin (black and brown hair) or pheomelanin (red and blond hair) [171,199]. Darker hair has higher melanin content resulting in greater drug binding. In this study, all participants had brown hair, except for M3 and M4 who had black hair. The highest concentration of mephedrone and NOR was detected in M3. This might suggest that the increased incorporation of these two analytes into hair could be due to larger eumelanin content. M4, the only other participant with black hair, did not show high concentrations of mephedrone or NOR which was likely due to the hair having purple highlights. The concentration of melanin was not determined but it might be a useful recommendation for future studies.

Metabolite to parent drug concentration ratios are one of the recommended criteria by the SoHT for differentiating external drug contamination from drug consumption. Contamination usually occurs through passive exposure to smoked drugs or drug powders and has been previously reported for cocaine [441], cannabis [442] and heroin [443]. Proposed metabolite to parent drug ratios indicative of cocaine ingestion are greater than 0.05 for the benzoylecgonine to cocaine ratio [215] and greater than 1.3 for the 6-monoacetylmorphine to morphine ratio in case of heroin consumption [443]. Recently, the ratio has also been proposed for MDMA and its metabolite, methamphetamine [444].

In this study, NOR:MEPH and DHNM:MEPH ratios were 0.19 (n=1) and 0.21 (n=1), respectively. The sample size is too small to suggest robust metabolite:MEPH cut-off levels, but this information may be useful in future research where a distinction between a drug user and a single dose administration could be made.

It is worth mentioning that current GC-MS and LC-MS analytical methods require extensive sample preparation which is time consuming and destructive to samples. Novel methods, such as MALDI-MS imaging [445] and DART-MS [251], have been used to identify analytes in an intact hair strand or a lock of hair. The use of MALDI-MS imaging for the analysis of intact strands of hair has been explored in collaboration with Sheffield Hallam University (data not shown here).

6.1.6 Conclusion

For the first-time mephedrone and its metabolites (NOR, DHM and DHNM) were detected in human head hair after a single intranasal dose of mephedrone hydrochloride. Metabolite to mephedrone ratios have also been reported for the first-time but a larger sample size is required before a useful cut-off level can be established.

6.2 Oral fluid

6.2.1 Detection of mephedrone and its metabolites in oral fluid

Analysis of oral fluid permits the detection of very recent drug use, and has increasingly been considered for use in the workplace and roadside drug testing [131]. Several analytical methods for quantification of mephedrone in oral fluid have been developed by GC-MS [372,373] and LC-MS [166,374,375], but only two of them analysed real biological samples. *Strano-Rossi et al.* published a LC-MS/MS screening method for the detection of new psychoactive substances in oral fluid [374]. When the method was applied to 400 real oral fluid samples from traffic control stops, mephedrone was not detected. In the

other study, *Mercolini et al.* detected mephedrone at 38 ng/mL and 15 ng/mL in unstimulated oral fluid collected by a disposable plastic pipette from self-reported drug users [166].

6.2.2 Oral fluid aims

The primary aim was to investigate the distribution of mephedrone and its metabolites in oral fluid. The secondary aim was to compare concentrations obtained in oral fluid with those obtained in whole blood/plasma in order to assess correlation between these two matrices.

6.2.3 Experimental

Oral fluid samples were extracted and analysed at Abbott (previously Alere Toxicology). All analytes were targeted as described before, except for two polar mephedrone metabolites, HYDROXY and 4-CARBOXY.

6.2.3.1 Reagents

In addition to the reagents described in Chapter 3 (Section 3.1.3.1), hydrochloric acid (HCl; 36%) was purchased from VWR (Lutterworth, UK). Certus® collection devices were provided by Abbott (previously Alere Toxicology).

6.2.3.2 Blank matrix collection

Blank oral fluid was collected from drug-free volunteers according to the ethical approval granted by the Research Ethics Committee at King's College London (HR 16/17 4237) which can be found in Appendix B.

Oral fluid was collected by the Certus® collection devices. Participants were asked to keep a swab under the tongue until the blue dye appeared indicating sufficient sample

volume. The swab was then transferred to a buffer solution (pH 6) which was shaken for 30 s. The swab was removed from the solution and buffered oral fluid was stored at -20°C until analysis.

6.2.3.3 Volunteer administration study and sample collection

Six healthy male volunteers nasally insufflated 100 mg of mephedrone hydrochloride. Oral fluid samples were collected as described in 6.2.3.2 at -10 min (0 h, before administration), 5 min, 15 min, 30 min, 60 min, 90 min, 2 h, 2.5 h, 5 h, Day 2 and Day 3. Insufficient volume of oral fluid was collected at 60 min (M2) and 90 min (M3) due to dry mouth. These samples were not analysed.

6.2.3.4 Working solutions

Working solutions used for the preparation of the calibration curve were prepared in methanol (MeOH) at 10, 20, 200, 1000, 2500, 5000, 10000 ng/mL for MEPH; 40, 160, 200, 1000, 2500, 5000, 10000 ng/mL for DHM; and 80, 200, 500, 1000, 2500, 5000, 10000 ng/mL for NOR and DHNM. Working solution used for the preparation of the quality control (QC) samples at low, medium and high level were made in MeOH at 10, 1000, 5000 ng/mL for MEPH; 80, 1000, 5000 ng/mL for DHM; and 170, 1000, 5000 ng/mL for NOR and DHNM. Internal standard (IS) solution containing MEPH-d_3, DHM-d_3 at 400 ng/mL was prepared in MeOH.

6.2.3.5 Calibration standards and quality control samples

Matrix-matched calibration standards containing MEPH at 0.5, 1, 10, 50, 125, 250, 500 ng/mL; DHM at 2, 8, 10, 50, 125, 250, 500 ng/mL; NOR and DHNM at 4, 10, 25, 50, 125, 250, 500 ng/mL were prepared by the addition of an appropriate volume of the working solution to buffered oral fluid. QC Low (0.5 ng/mL for MEPH; 2 ng/mL for DHM; 7 ng/mL for NOR and DHNM), QC Med (50 ng/mL for MEPH, DHM, NOR, DHNM) and QC

High (250 ng/mL for MEPH, DHM, NOR, DHNM) were prepared by the addition of an appropriate volume of the working solution to buffered oral fluid.

Calibration standards and QCs were prepared fresh on the day of sample analysis. Blanks containing buffered oral fluid but no IS and one sample containing buffered oral fluid and IS were also prepared and taken through the extraction.

6.2.3.6 Sample preparation

Four hundred microliters of buffered oral fluid was extracted using SPE. Twenty microliters of the IS was added to buffered oral fluid, calibration standards and QCs. Twenty microliters of MeOH was added to the blanks. All samples were vortex mixed and 200 µL of 0.1 M HCl:MeOH (50:50 v/v) was added. After conditioning a mixed mode MCX µElution plate with 200 µL of MeOH and 200 µL of 0.1 M $HCl_{(aq)}$, samples were loaded and washed with 200 µL of 0.1 M $HCl_{(aq)}$ followed by 200 µL of ACN:water (30:70 v/v). Samples were eluted with 4 x 25 µL of MeOH:NH_4OH (97:3 v/v) and dried under nitrogen at 25°C. Samples were reconstituted with 50 µL of 0.1% formic acid in water. Sample dilution was not require.

6.2.3.7 LC-MS/MS conditions

The analysis was performed on Q-Exactive Focus Orbitrap MS (Thermo Scientific, UK) coupled to UltiMate 3000 (Thermo Scientific, UK) UPLC system. Extracted samples were analysed in full scan and MS/MS mode using a heated electrospray ionisation source (HESI-II) operated in positive ion mode. Full scans were acquired from m/z 100 to 750 with 70,000 FWHM (full width at half-maximum, scan speed 3 Hz) resolution with the automatic gain control (AGC) target set to 1×10^6. S-lens radio frequency was set to 25 V, skimmer voltage was 15 V and the spray voltage was 3.75 kV. Auxiliary gas temperature was kept at 375°C, capillary temperature was 320°C, sheath gas flow rate was 50 and the auxiliary gas flow rate was 5. In the MS/MS mode the instrument was operated using an inclusion list containing appropriate precursor ions, an optimised collision energy of

60 eV and a product ion scan range of m/z 50 to 200. The mass spectrometer acquired MS/MS scans at a resolution of 17,500 FWHM with the AGC target of 5×10^5.

Chromatographic separation was performed using a Thermo Accucore C18 column (100 x 2.1 mm, 2.6 µm) held at 40°C. The needle wash was MeOH:water (50:50 v/v). The flow rate was 0.5 mL/min with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in ACN as mobile phase B. The start of the gradient was at 95% mobile phase A and was kept for 2 min. Mobile phase B was then increased to 95% over 2 min and was held for 0.5 min. Over the next 0.1 min the gradient returned to the starting condition and the column was re-equilibrated at 95% mobile phase A for the remaining 0.4 min. The total run time was 5 min. The injection volume was 10 µL and the data was acquired using Xcalibur® software (version 14.0).

6.2.3.8 Pharmacokinetic calculations

Please refer to Section 2.3 in Chapter 2.

6.2.3.9 Validation procedure

Please refer to Section 2.5 in Chapter 2.

6.2.4 Results

6.2.4.1 Method validation

Please refer to in Section 2.6.5 in Chapter 2.

6.2.4.2 Concentrations of mephedrone and its metabolites in oral fluid

Mean oral fluid concentrations ± SD for mephedrone and NOR in 6 participants are shown in Figure 6-3 and Table 6-2, respectively. Dihydro-mephedrone (DHM) and dihydro-nor-mephedrone (DHNM) were not detected in the samples.

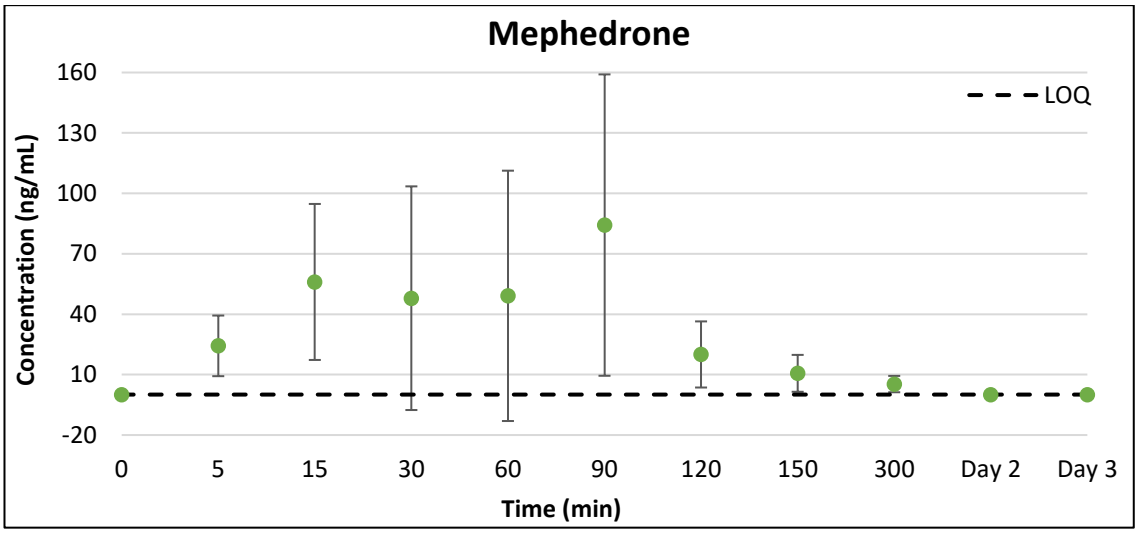


Figure 6-3. Mean mephedrone oral fluid concentrations ± SD (n=6); the black dashed line represents the limit of quantification (LOQ)

Following the administration, mephedrone was first detected in oral fluid after 5 min, peaked at 90 min and was undetectable on Day 2 and Day 3. Error bars at 30 min and 60 min were disproportionally large because mephedrone concentrations in M1 and M2 were approximately 4 times higher compared to other participants.

Interestingly, the elimination profile for M5 and M6 showed a decrease in mephedrone concentration at 30 min followed by an increase at 90 min before the concentrations declined again at 120 min (Figure 6-4).

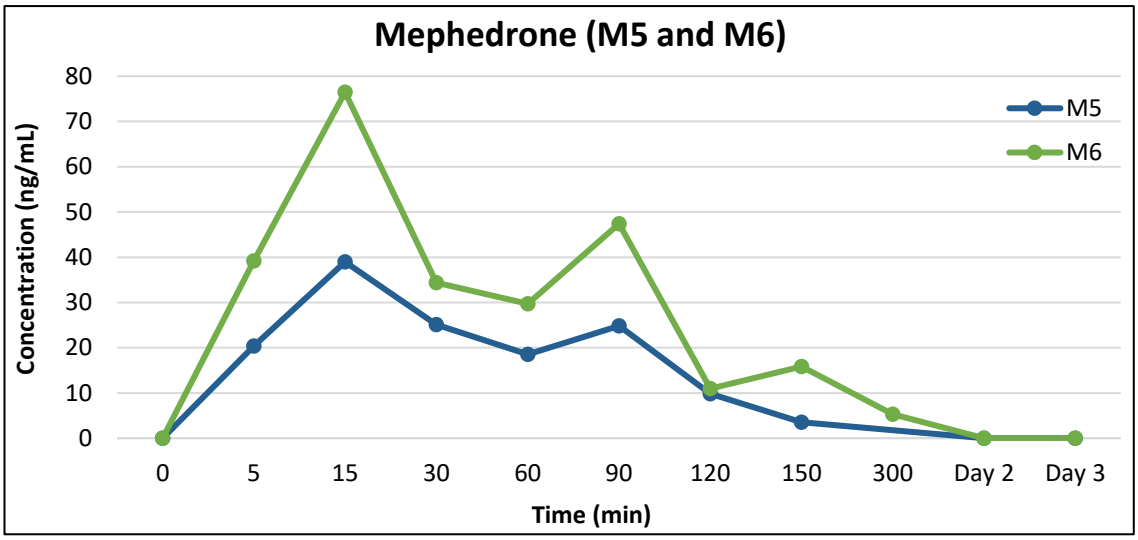


Figure 6-4. Mephedrone concentrations in oral fluid in M5 and M6

As shown in Table 6-2, NOR was detected in 33 (56.9%) oral fluid samples above the LOQ (4 ng/mL) and in 45 (77.6%) oral fluid samples above the LOD (1 ng/mL). NOR was detected above the LOD between 5 min and 300 min, except in M5 where the analyte was first detected after 15 min. Mean NOR concentration (12.8 ± 2.0 ng/mL) peaked at 90 min and was undetectable on Day 2 and Day 3.

Table 6-2. Nor-mephedrone concentrations (expressed in ng/mL) above the LOQ of 4 ng/mL and LOD (ticks) in oral fluid for M1-M6 at each timepoint; NA - sample not collected due to dry mouth; ND - not detected

Timepoints (min)	M1	M2	M3	M4	M5	M6	Mean ± SD
5	10.1	✓	10.3	✓	ND	✓	10.2 ± 0.1
15	11.7	✓	11.3	✓	✓	✓	11.4 ± 0.3
30	12.4	11.8	11.6	✓	11.1	11.1	11.6 ± 0.5
60	13.8	NA	11.2	10.2	11.2	11.4	11.6 ± 1.3
90	16.2	13.1	NA	11.3	11.2	12.3	12.8 ± 2.0

120	12.8	11.4	11.0	11.5	11.6	11.3	11.6 ± 0.6
150	12.2	11.9	10.2	✓	10.2	11.7	11.2 ± 1.0
300	10.8	✓	✓	✓	✓	10.4	10.6 ± 0.3
Day 2	ND	ND	ND	ND	ND	ND	-
Day 3	ND	ND	ND	ND	ND	ND	-

6.2.4.3 Pharmacokinetic analysis

Oral fluid drug concentrations were fitted with a single-dose, first-order elimination phase model and the calculated mean pharmacokinetic (PK) parameters are summarised in Table 6-3. Pharmacokinetic parameters (other than C_{max} and T_{max}) were not determined for NOR because an insufficient number of datapoints was observed from the elimination phase. Mean C_{max} and T_{max} for NOR were 12.7 ± 1.8 ng/mL and 90 ± 33 min, respectively.

Table 6-3. Mean ± SD mephedrone pharmacokinetic data from the analysis of oral fluid samples from 6 male participants

	C_{max} (ng/mL)	**T_{max}** (min)	**k_{el}** (min^{-1})	**$t_{1/2}$** (h)	**AUC** (ng mL^{-1} h)	**CL** (mL min^{-1} kg^{-1})	**V** (L kg^{-1})
M1	164	90	0.047	0.25	303	65.2	1.39
M2	167	90	0.006	2.00	213	103	17.8
M3	22.7	15	0.011	1.05	34.2	705	68.5
M4	68.5	15	0.011	1.05	62.6	449	41.0
M5	39.0	15	0.023	0.50	47.6	424	18.4
M6	76.0	15	0.007	1.65	108	279	39.8
Mean ± SD	89.6 ± 61.9	40.0 ± 38.7	0.017 ± 0.016	1.09 ± 0.64	128 ± 108	345 ± 254	31.1 ± 23.6

Mephedrone reached the mean C_{max} of 89.6 ± 61.9 ng/mL at 40.0 ± 38.7 min, with both M1 and M2 showing higher C_{max} and delayed T_{max}. With regards to elimination, mephedrone had mean k_{el} of 0.017 ± 0.016 min^{-1} and mean $t_{1/2}$ of 1.09 ± 0.64 h. The smallest AUC was recorded for M3 (34.2 ng mL^{-1} h) which was approximately 9 times smaller than the largest AUC (303 ng mL^{-1} h) recorded for M1. Mean V for mephedrone was 31.1 ± 23.6 L kg^{-1} and CL was 345 ± 254 mL min^{-1} kg^{-1}. M1 had the smallest V and CL of 1.39 L kg^{-1} and 65.2 mL min^{-1} kg^{-1}, respectively, while M3 had the largest V of 68.5 L kg^{-1} and CL of 705 mL min^{-1} kg^{-1}.

6.2.4.4 Comparison of the pharmacokinetic parameters in oral fluid, plasma and whole blood

As shown in Table 6-4, mephedrone concentrations peaked earlier in oral fluid (40.0 ± 38.7 min) compared to whole blood (55.0 ± 18.2 min) and plasma (52.5 ± 20.7 min). Even though delayed T_{max} was observed in whole blood and plasma, similar C_{max} values were obtained in all matrices. Mephedrone was eliminated with $t_{1/2}$ of approximately 2 h in whole blood/plasma (k_{el} of 0.006 ± 0.001 min^{-1}) compared to 1.09 ± 0.64 h (k_{el} of 0.017 ± 0.016 min^{-1}) in oral fluid. A smaller mean AUC was calculated in oral fluid compared to whole blood (54.1 ± 15.9 mL min^{-1} kg^{-1}) and plasma (66.5 ± 23.6 mL min^{-1} kg^{-1}). Larger CL and V were also observed in oral fluid.

Pharmacokinetic parameters, other than C_{max} and T_{max}, were not determined for NOR in oral fluid. The analyte peaked at 90 ± 33 min compared to 133 ± 27.5 min in whole blood and 130 ± 35.1 min in plasma. NOR reached the highest C_{max} of 12.7 ± 1.8 ng/mL in oral fluid in comparison with 5.12 ± 2.16 ng/mL in whole blood and 7.87 ± 5.35 ng/mL in plasma.

Table 6-4. Comparision of mephedrone pharmacokinetic parameters between whole blood, plasma and oral fluid (expressed as mean ± SD)

Matrix	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)	CL (mL min^{-1} kg^{-1})	V (L kg^{-1})
Whole blood	101 ± 45.4	55.0 ± 18.2	0.006 ± 0.001	2.12 ± 0.33	474 ± 150	54.1 ± 15.9	9.91 ± 2.96
Plasma	89.8 ± 41.7	52.5 ± 20.7	0.006 ± 0.001	1.98 ± 0.30	395 ± 144	66.5 ± 23.6	11.4 ± 4.5
Oral fluid	89.6 ± 61.9	40.0 ± 38.7	0.017 ± 0.016	1.09 ± 0.64	128 ± 108	345 ± 254	31.1 ± 23.6

6.2.4.5 Oral fluid correlation with whole blood

Correlation between analyte concentrations detected in oral fluid and whole blood has been investigated for each participant as shown in Figure 6-5 and Figure 6-6 (the black dotted line shows a trend line). Individual correlation can be found in Appendix E.

Mephedrone did not correlate well between the two matrices as evidenced by the Pearson correlation coefficient of 0.361 whereas NOR showed moderate correlation with the Pearson correlation coefficient of 0.437.

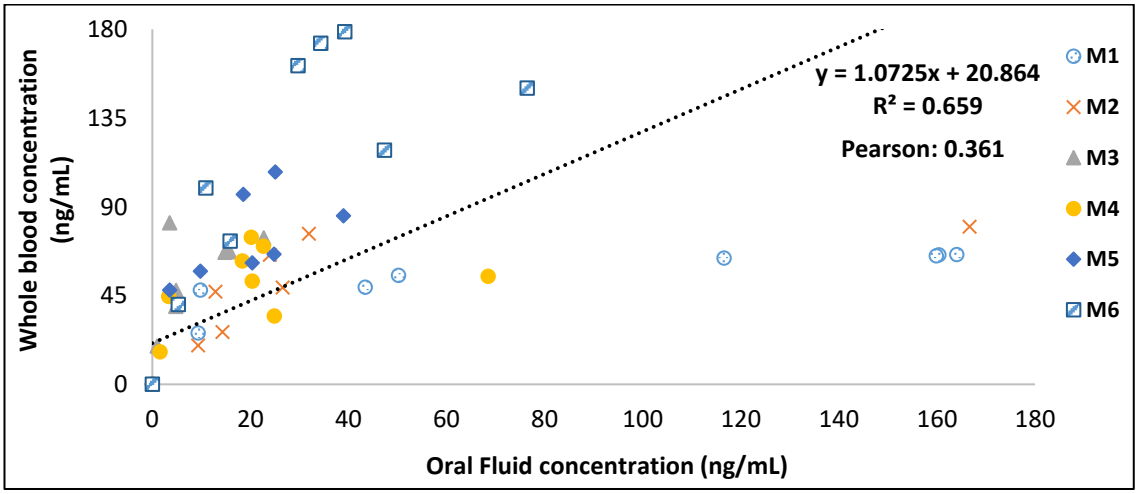


Figure 6-5. Correlation of oral fluid and whole blood concentrations for mephedrone (n=6)

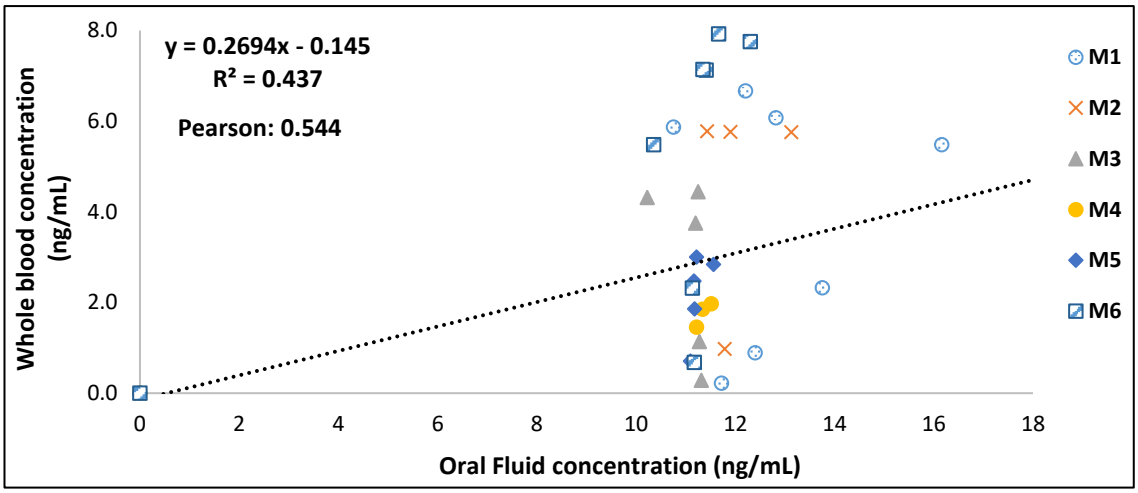


Figure 6-6. Correlation of oral fluid and whole blood concentrations for nor-mephedrone (n=6)

6.2.4.6 Oral fluid correlation with plasma

Correlation between analyte concentrations detected in oral fluid and plasma has been investigated for each participant as shown in Figure 6-7 and Figure 6-8 (the black dotted line shows a trend line). Individual correlation can be found in Appendix E.

Mephedrone did not correlate well between the two matrices as evidenced by the Pearson correlation coefficient of 0.286 whereas NOR showed better correlation with the Pearson correlation coefficient of 0.608.

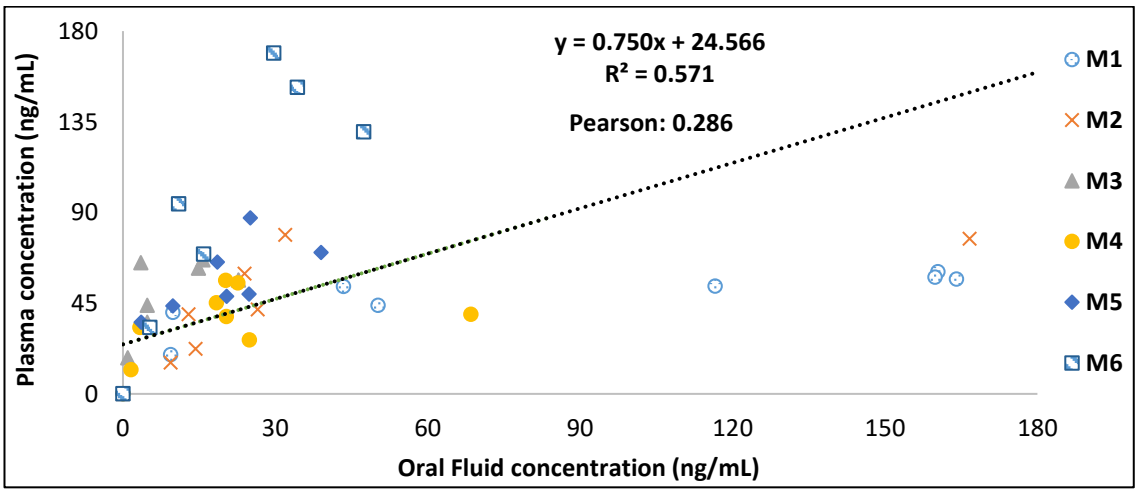


Figure 6-7. Correlation of oral fluid and plasma concentrations for mephedrone (n=6)

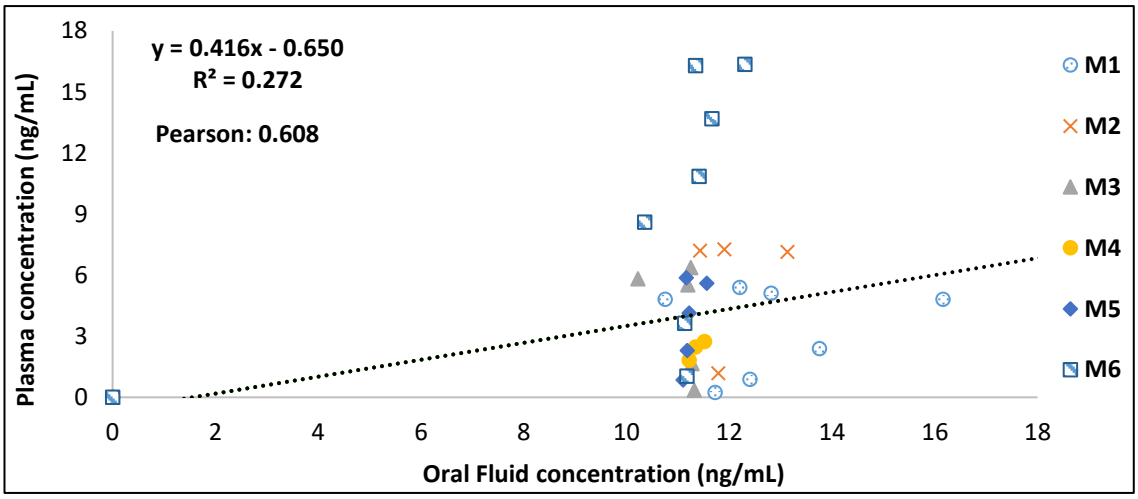


Figure 6-8. Correlation of oral fluid and plasma concentrations for nor-mephedrone (n=6)

6.2.4.7 Whole blood and oral fluid – method comparison

Bland-Altman analysis was performed to examine the agreement between concentrations obtained for whole blood and oral fluid. Bland-Altman plots (Figure 6-9 and Figure 6-10) show the difference between paired concentrations from oral fluid and whole blood samples plotted against the mean of the two concentrations calculated for each individual sample. Dotted lines represent the 95% limits of agreement (mean difference ± 2 SD).

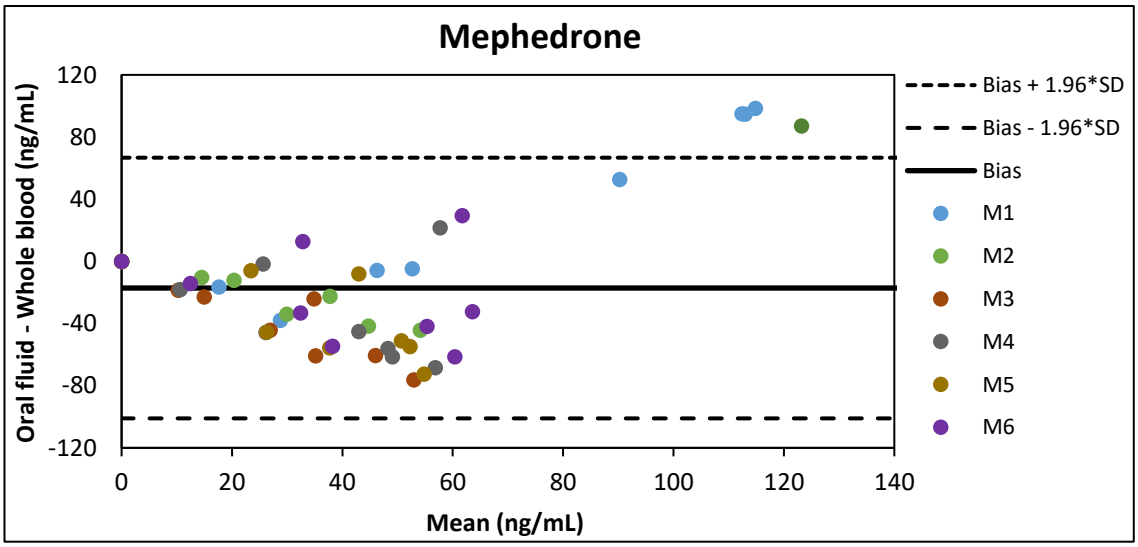


Figure 6-9. Bland-Altman plots comparing whole blood and oral fluid methods for mephedrone

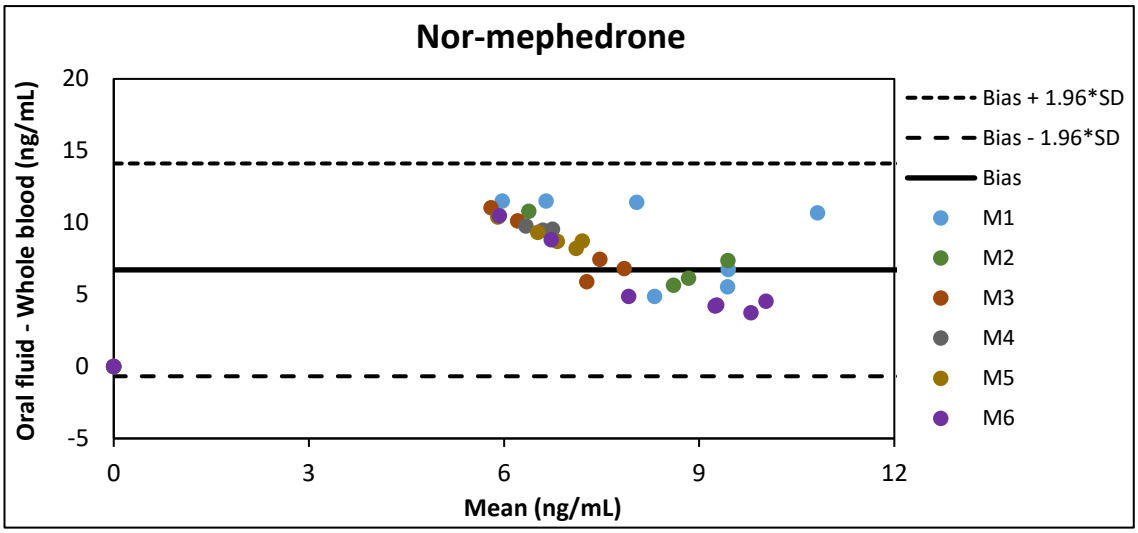


Figure 6-10. Bland-Altman plots comparing whole blood and oral fluid methods for nor-mephedrone

Bland-Altman plots showed bias ± SD between whole blood and oral fluid concentrations to be -17.2 ± 42.8 ng/mL for mephedrone and 6.72 ± 3.80 ng/mL for NOR. Whole blood NOR concentrations were in good agreement with oral fluid concentrations with > 95% of datapoints being within ± 2 SD of the bias. The agreement was poorer for mephedrone with only 92.2% of datapoints being within ± 2 SD of the bias.

6.2.4.8 Plasma and oral fluid – method comparison

Bland-Altman analysis was performed to examine the agreement between concentrations obtained for plasma and oral fluid. Bland-Altman plots (Figure 6-11 and Figure 6-12) show the difference between paired concentrations from oral fluid and plasma samples plotted against the mean of the two concentrations calculated for each individual sample. Dotted lines represent the 95% limits of agreement (mean difference ± 2 SD).

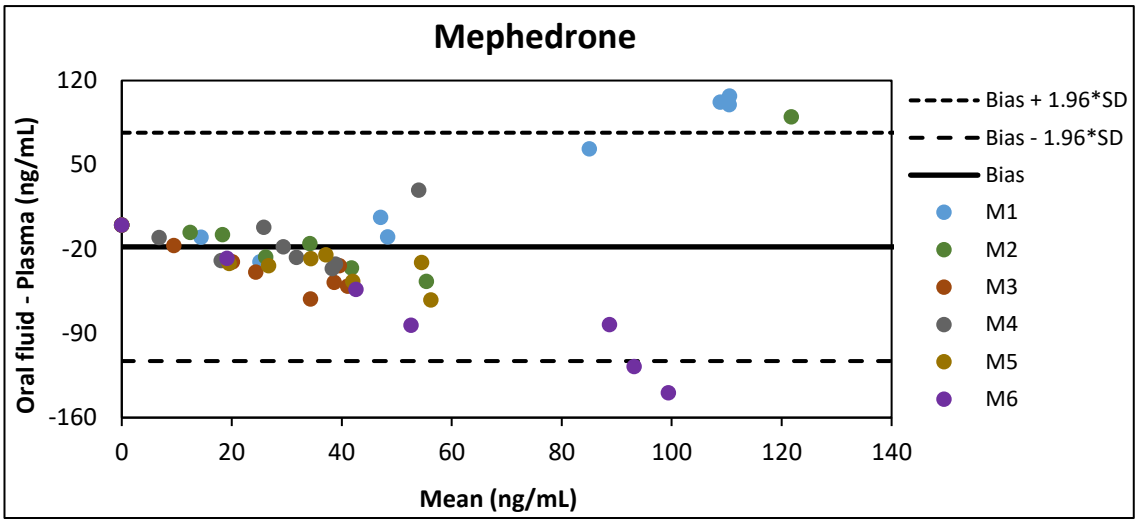


Figure 6-11. Bland-Altman plots comparing plasma and oral fluid methods for mephedrone

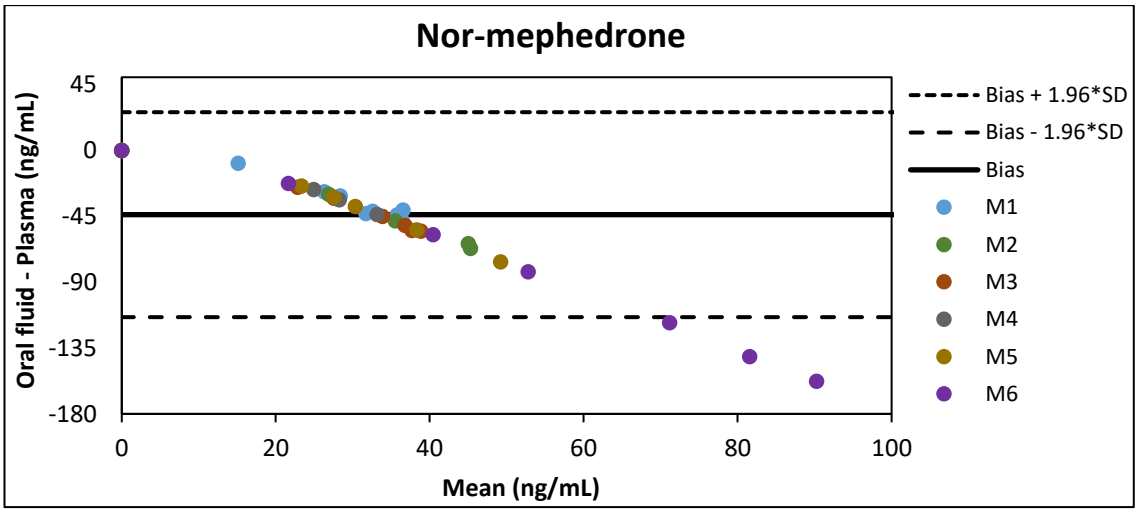


Figure 6-12. Bland-Altman plots comparing plasma and oral fluid methods for nor-mephedrone

Bland-Altman plots showed bias ± SD between plasma and oral fluid concentrations to be -18.2 ± 48.4 ng/mL for mephedrone and -43.9 ± 35.7 ng/mL for NOR.. 87.8% and 92.1% of datapoints associated with mephedrone and NOR were within ± 2 SD of the bias, respectively.

6.2.5 Discussion

Mephedrone and NOR concentrations peaked earlier in oral fluid compared to whole blood and plasma which may have been due to the buccal contamination with mephedrone following nasal insufflation. Moreover, significantly faster clearance of mephedrone in oral fluid resulted in a shorter detection window compared to whole blood and plasma. Mephedrone was detected in five participants up to 5 h after administration in oral fluid but was undetectable on Day 2 and Day 3. This is in contrast with whole blood and plasma where mephedrone was detected in all participants up to 6 h and in one participant on Day 2 in whole blood.

Drugs of abuse usually contain an ionisable amine group which is characterised by a pKa value greater than the pH of oral fluid (6.7-7.4) [131]. Once a basic drug gets incorporated into oral fluid, it becomes ionised which prevents its back diffusion into the blood (pH 7.4). Basic drugs will, therefore, be present for longer and at higher concentrations in oral fluid than acidic drugs, such as cannabis (THC and its carboxy metabolite) [137]. DHM (pKa = 9.55) and DHNM (pKa = 9.39) should in theory get incorporated into oral fluid but were not detected in this study. The reason for that may be the fact that DHM and DHNM are minor metabolites of mephedrone. In whole blood DHM and DHNM represented only 3.38% and 1.69% of the AUC of mephedrone, respectively (see Section 3.1.4.3 in Chapter 3 for more details). In addition, a relatively small dose of 100 mg of intranasally administered mephedrone might have resulted in DHM and DHNM not being detected in oral fluid under the reported experimental conditions.

Potential contamination of the oral cavity following smoking, snorting or swallowing drugs can in general be observed for several hours after drug intake. In this study mephedrone was administered via nasal insufflation which might have resulted in mephedrone particles clinging to the mucus membranes of the nostrils and turbinates where direct vascular absorption took place. In addition, smaller particles of mephedrone might have travelled deeper into the respiratory tract where they got

trapped by the mucus present in nasopharynx, trachea, bronchi and terminal bronchioles. As a result, respiratory contamination might have contributed to the detected mephedrone concentrations in oral fluid [169]. It is difficult to determine the extent of oral fluid contamination, but disproportionally large error bars at 30 min and 60 min in M1 and M2 were observed for mephedrone.

Inter-individual variation in detected mephedrone concentrations was also observed. As this is the first controlled mephedrone administration study targeting mephedrone and its metabolites in oral fluid, it is not possible to compare the level of variability. However, inter-individual variations in oral fluid samples were reported in other controlled administration studies of cocaine [151,169], codeine/norcodeine [446] and amphetamine/methamphetamine [152]. The variation could be due to the efficiency of mephedrone absorption after nasal insufflation, the small number of participants, differences in oral fluid pH (not measured in this study) and the rate of oral fluid production. In M5 and M6 different elimination profiles were observed, with a decrease in mephedrone concentration at 30 min followed by an increase at 90 min before the concentrations declined again at 120 min. The spike in mephedrone concentration at 15 min and 90 min could have been due to contamination of the oral cavity after taking the drug or the residual mephedrone in the nasal cavity being re-absorbed.

The Pearson correlation coefficient indicated moderate correlation between NOR concentrations in oral fluid and both whole blood (Pearson = 0.437) and plasma (Pearson = 0.608). Poor agreement was observed for mephedrone between oral fluid concentrations and whole blood as well as plasma concentrations as evidenced by the Pearson correlation coefficient of 0.361 and 0.286, respectively. Bland-Altman analysis showed that more than 95% of the observed NOR concentrations were within ± 2 SD of the bias in whole blood but not in plasma. In addition, less than 95% of the observed mephedrone concentrations were within ± 2 SD of the bias in plasma and whole blood. Negative bias of -17.2 ng/mL and -18.2 ng/mL was observed for mephedrone in whole blood and plasma, respectively, suggesting oral fluid concentrations were

overestimated. NOR showed positive bias in whole blood (6.72 ng/mL) and negative bias in plasma (-43.9 ng/mL), suggesting oral fluid concentrations were underestimated in whole blood and overestimated in plasma. Interestingly, poorer correlation was observed at higher concentrations which might be linked to the oral contamination following intranasal administration of mephedrone. Poor correlation may also be a result of protein binding which affects drug distribution and elimination. The extent of mephedrone or its metabolites binding to plasma proteins has not been determined in humans but 21.6 ± 3.67% of mephedrone has been shown to bind to proteins in Sprague-Dawley rats [92].

6.2.6 Conclusion

This is the first-time mephedrone and one of its metabolites, nor-mephedrone, were detected in oral fluid samples after a controlled administration study. Poor to moderate correlation between oral fluid and whole blood/plasma analyte concentrations was observed with Bland-Altman analysis showing wide confidence levels demonstrating poor agreement between the methods. However, poor correlation might be linked to the contamination of the oral cavity with mephedrone or analyte binding to plasma proteins.

CHAPTER 7

OVERALL CONCLUSIONS AND FUTURE WORK

7.1 Summary

7.1.1 Background

In recent years, there has been an increasing interest in the use of alternative biological matrices, such as oral fluid, dried blood spots (DBS), head hair, breath and sweat for determining drug use. The collection of these samples is usually non-invasive, fast and cost effective which allows for drug testing in the workplace, by the roadside or in addiction treatment centres. Moreover, alternative biological matrices are usually easier and cheaper to transport than the conventional biological matrices, and some of them (head hair, DBS in some cases) can be stored at ambient conditions.

Mephedrone is a synthetic cathinone known for its psychostimulant properties [30–32]. Synthetic or substituted cathinones, which are chemically derived from an alkaloid naturally present in the leaves of *Catha edulis* (Khat), are one of the biggest groups of new psychoactive substances. Even though mephedrone use has declined since its ban in April 2010 in the UK, there is evidence that its use in London remains popular [63] and there are reports describing an increasing problem of people injecting the drug [64]. Nevertheless, there is little known about the distribution of mephedrone and its metabolites in the alternative and conventional biological matrices as only two controlled mephedrone administration studies [91,97,414] and one dose-finding pilot study [75] have been conducted.

7.1.2 Methodologies

A single dose administration study of 100 mg mephedrone hydrochloride via nasal insufflation to six healthy male volunteers was performed to determine the distribution and pharmacokinetics of mephedrone and its metabolites in conventional and alternative biological matrices. Samples were collected at different timepoints after mephedrone administration and were analysed for the presence of mephedrone, dihydro-mephedrone (DHM), nor-mephedrone (NOR), hydroxytolyl-mephedrone (HYDROXY), 4-carboxy-mephedrone (4-CARBOXY) and dihydro-nor-mephedrone (DHNM) by validated LC-MS/MS methods.

7.1.3 Conclusions

Fully validated methods for the quantification of mephedrone and five of its Phase I metabolites have been developed and successfully applied to the analysis of samples collected during the controlled mephedrone administration. The development of these methods took up to two years and was challenging for a number of reasons. Simultaneous extraction and chromatographic separation of the analytes with a wide range of polarities required time-consuming method optimisation. Problems were also encountered during the development of the chiral method aimed at separating mephedrone enantiomers. Unsuccessful work with various chiral columns on supercritical fluid chromatography was followed by an extensive evaluation of derivatisation reagents and other analytical methods until a satisfactory separation was achieved.

7.1.3.1 Sample analysis

Plasma samples have been previously collected and analysed after an oral administration of mephedrone but to my knowledge there are no reported studies describing concentrations of mephedrone and five of its Phase I metabolites in whole blood or in plasma after nasal insufflation of mephedrone. In this study, all analytes were

detected in whole blood and plasma, where 4-CARBOXY reached the highest concentration. NOR was the second most abundant metabolite whereas DHNM had the lowest C_{max} in both matrices. This is in agreement with a previously published oral mephedrone administration study [91] which reported that 4-CARBOXY was the predominant metabolite detected in plasma followed by NOR. Moreover, pharmacokinetic parameters corresponded well between whole blood and plasma and a previously published half-life from orally administrated mephedrone. As expected, T_{max} was shorter following 100 mg of mephedrone given intranasally compared with oral doses of up to 200 mg. In addition, statistical analysis showed that median whole blood to plasma distribution ratios, reported here for the first time, were statistically different from 1 (unity) for mephedrone (median: 1.11), DHM (median: 1.30) and NOR (median: 0.765). In DBS mephedrone, NOR and 4-CARBOXY were the only analytes detected in the majority of samples. Interestingly, capillary NOR concentrations were higher than the concentrations measured in whole blood and plasma. Pharmacodynamics was also evaluated by monitoring changes in subjective and physiological effects following mephedrone administration. Stimulant-like cardiovascular effects were manifested by increases in heart rate, systolic blood pressure and diastolic blood pressure. Mephedrone also produced increases in several visual analogue scale parameters related to stimulant-like effects and changes in perception.

This is the first study that reports R-mephedrone (R-MEPH) and S-mephedrone (S-MEPH) concentrations in whole blood and investigates their pharmacokinetics after a controlled administration. R-MEPH reached higher concentrations than S-MEPH and had comparable pharmacokinetic parameters to total mephedrone. These results are in agreement with previous research which found R-MEPH to be a predominant analyte in pooled human urine samples collected from a music festival and in *in vitro* experiments with human liver microsomes [87]. This research shows that the two enantiomers of mephedrone exhibit different pharmacokinetic profiles in humans, but the clinical significance of this finding is not yet fully understood.

A sensitive LC-MS method targeting mephedrone and its metabolites in head hair allowed the detection of the analytes at low pg/mg level. It has been shown for the first-time that mephedrone metabolites (NOR, DHM and DHNM) can be detected in human head hair one month after mephedrone administration. Moreover, metabolite to mephedrone ratios, which are used to differentiate external drug contamination from drug consumption, have been evaluated for the first-time. Calculated NOR:mephedrone and DHNM:mephedrone ratios were 0.19 (n=1) and 0.21 (n=1), respectively. The sample size was too small to suggest robust metabolite to mephedrone ratio cut-off levels, but this information may be useful in future research where a distinction between a drug user and a single dose administration could be made.

Another novel aspect of the project focused on determination of mephedrone and its metabolites concentrations in fingerprint sweat and oral fluid. Mephedrone and NOR were detected above the limit of detection in 62% and 3.8% of all post administration samples, respectively. Inter- and intra-subject variability was observed, which can be attributed to the differences in pressure applied during fingerprint deposition, the angle and duration of contact with the deposition surface coupled with the inability to control the 'amount' of collected sweat. Given these limitations fingerprint sweat may not be ideal for use in quantitative analysis until practical solutions to these problems are found. In oral fluid mephedrone, DHM, NOR and DHNM were targeted but only mephedrone and NOR were detected in the majority of samples. Mephedrone and NOR concentrations peaked earlier in oral fluid compared to whole blood and plasma which may be due to the buccal contamination with mephedrone following nasal insufflation.

7.1.3.2 Correlation

The relationship between analyte concentrations in whole blood, plasma, DBS and oral fluid was investigated to further explore the viability of alternative matrices for the detection of drugs and their metabolites. Good to moderate correlation was observed for the analytes, which might have also been affected by CYP2D6 polymorphism, plasma

protein binding and, in case of oral fluid, contamination of the oral cavity with mephedrone.

7.1.3.3 Window of detection

The window of detection, which describes how long drugs can be detected in a biological sample above a cut-off level, is an important consideration in forensic toxicology. Many factors influence the window of detection, such as the frequency of drug use (chronic or acute), amount taken, rate of metabolism, body fat or medical conditions (liver or kidney disease) which may influence metabolism/elimination. Drugs are usually detected in urine for longer than in whole blood/plasma whereas head hair provides a historic record of drug use, with the detection window being dependent on hair length.

In this study, analytes exhibited a wide range of detection windows. As seen in Figure 7-1, 4-CARBOXY and DHNM were the only metabolites detectable in the majority of urine samples on Day 3, making them promising markers of mephedrone use. This is a crucial finding for the clinical and forensic toxicologists who by targeting 4-CARBOXY and DHNM in urine may be able prove mephedrone use. Moreover, by selectively targeting mephedrone and its metabolites in biological matrices exhibiting different detection windows it might be possible to determine the time of mephedrone use. In head hair, mephedrone and NOR had the longest detection windows, with the analytes being detected 1 month after administration. On the other hand, HYDROXY had the shortest detection window as it was detectable in whole blood, plasma and urine for only up to 6 h. However, it needs to be re-emphasised that with a more sensitive method and a higher dose of administered mephedrone, which would be more representative of the amount mephedrone users report to take, longer detection windows may be achieved for some analytes.

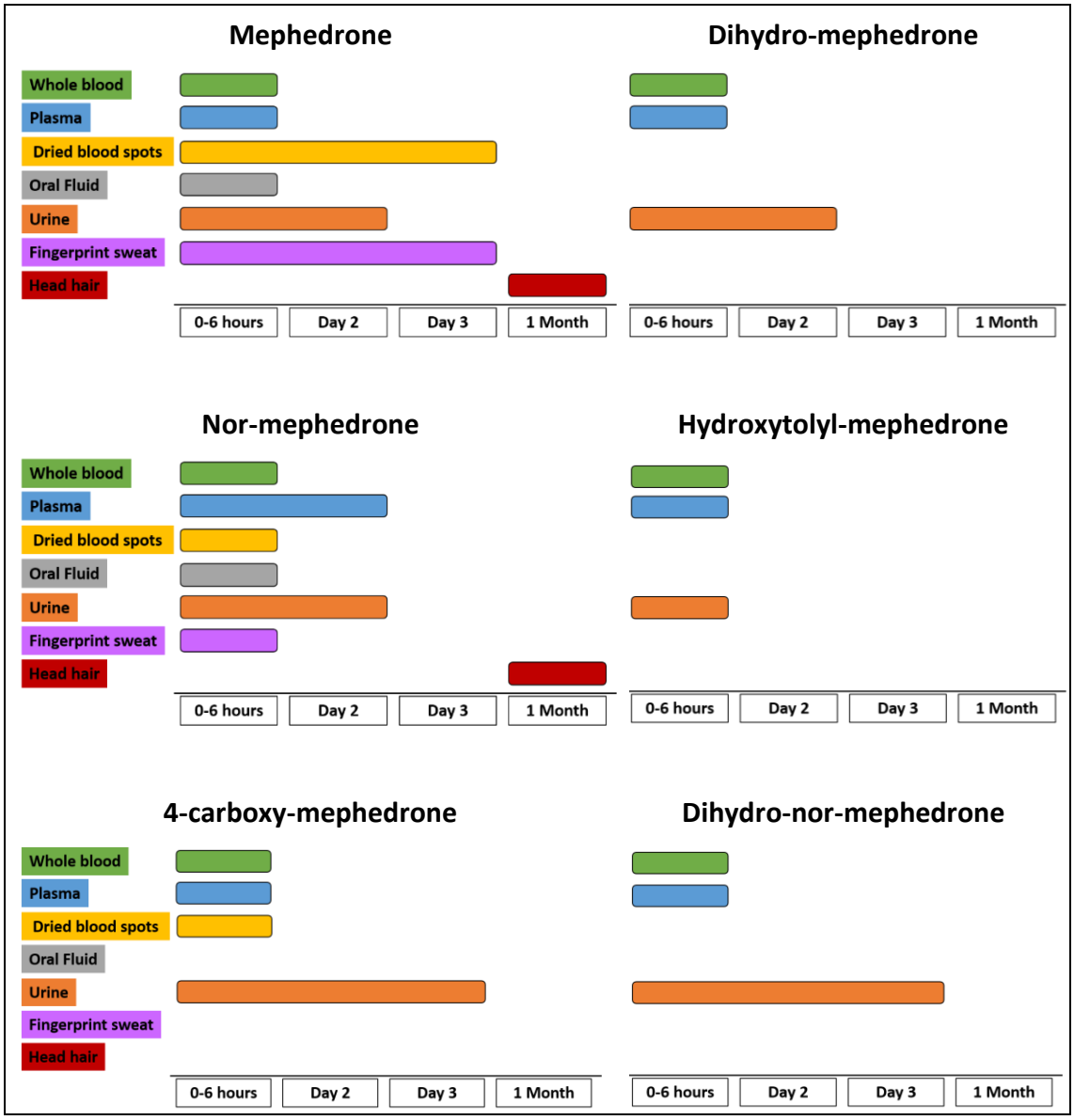


Figure 7-1. Comparison of the detection windows for each analyte detected in the majority of samples analysed in the study

7.2 Future work

Due to time constraints, breath and head sweat samples collected during the administration study were not analysed. In the future, it would be beneficial to develop and validate suitable analytical methods for quantification of mephedrone and its metabolites in these two matrices.

Low detectability of mephedrone and its metabolites in several biological matrices, such as fingerprint sweat and oral fluid, has been partially attributed to a single and relatively low dose of administered mephedrone. Future research would benefit from conducting a controlled mephedrone administration study with higher and/or repeated doses and larger sample size. The latter would also help minimise inter-subject variability which was observed in the study. Another cause of variability might be CYP2D6 genetic polymorphism. Buccal swabs for CYP2D6 genotyping were collected from the participants in this study but were not analysed in time for the thesis submission.

Several international and national collaborations have been established during this PhD project. Plasma samples were sent to the University of Zurich for metabolomics analysis, hair strands were analysed by MALDI-MS imaging in Sheffield Hallam University, fingerprint sweat samples collected on triangular pieces of chromatography paper were analysed by paper spray at University of Surrey and DBS collected on filter paper were sent for analysis to Ghent University. All collaborations are briefly mentioned in the thesis and the results will be submitted for publication in due course.

It has been shown that mephedrone enantiomers exhibit different pharmacokinetic profiles in humans which might be a result of pharmacokinetic processes occurring at different rates during drug absorption, distribution, metabolism or excretion. The mechanism responsible for the difference in concentrations has not been explored and warrants further work. In addition, clinical significance of this finding is not yet fully understood. So far neurochemical and behavioural effects of R-MEPH and S-MEPH have only been investigated by a receptor binding assay [447] and *in vivo* in rats [86]. The next useful step would be to conduct a controlled study involving an administration of each enantiomer to human subjects. Such a study would benefit from a large sample size split into a control (placebo) and treatment (R-MEPH and S-MEPH administration) groups. Ethical approval would have to be obtained and the enantiomers of mephedrone would have to be synthesised in a laboratory with an appropriate licence because they are not currently available for purchase.

Even though mephedrone and its metabolites have been detected and quantified in samples collected from controlled administration studies, more translational research involving the analysis of clinical and forensic samples is needed. To my knowledge only one other study has reported the presence of mephedrone metabolites (DHM, NOR, HYDROXY and 4-CARBOXY) in urine and blood samples collected from road traffic cases [70]. It is especially important to target NOR which has been shown to be an active metabolite able to cross the blood-brain barrier and contribute to the psychostimulant effects of mephedrone [91]. Lastly, metabolites which have demonstrated longer windows of detection should be targeted in clinical and forensic studies where they could be used as markers of mephedrone use.

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APPENDIX A

Mephedrone administration - ethical approval letter

Health Research Authority

London - Riverside Research Ethics Committee
Level 3 Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Telephone: 0207 104 8037

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

08 September 2016

Prof Paul Dargan
Guy's and St Thomas' Hospital, NHS Foundation Trust
Westminster Bridge Road
London
SE1 7EH

Dear Prof Dargan

Study title:	**Mephedrone: a single-dose administration study to determine human pharmacokinetics after nasal insufflation and to detect mephedrone and its metabolites in novel biological matrices**
REC reference:	**16/LO/1342**
IRAS project ID:	**202317**

Thank you for your letter of 25th August 2016, responding to the Committee's request for further information on the above research.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Tina Cavaliere, nrescommittee.london-riverside@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

You should notify the REC once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Revised documents should be submitted to the REC electronically from IRAS. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which you can make available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [KCL study advertisement]	Version 3.0	01 September 2016
IRAS Application Form [IRAS_Form_04092016]		04 September 2016
IRAS Application Form XML file [IRAS_Form_04092016]		04 September 2016
IRAS Checklist XML [Checklist_04092016]		04 September 2016
Letter from funder [BBSRC funding letter]		07 May 2015
Other [Glossary of Terms and Abbreviations]	Version 1.0	08 March 2016
Other [Mephedrone assessment form]	Version 1.0	23 November 2015
Other [Table and Figures]	Version 1.0	08 March 2016
Other [Confirmation that the study is not a CTIMP]	Version 1.0	17 May 2016
Other [16 LO 1342 Response to the provisional opinion]	Version 1.0	23 August 2016
Other [Mephedrone newspaper advertisment]	Version 3.0	01 September 2016
Other [Support Letter Mephedrone Study]	Version 1	30 May 2016
Other [Alere Toxicology funding letter]	Version 1	03 June 2016
Other [Controlled Drug Licence]	Version 1	23 August 2016
Other [Email from the Home Office about the licence]	Version 1	23 August 2016
Participant consent form [Mephedrone participant consent form]	Version 1.0	23 November 2015
Participant information sheet (PIS) [Participant information leaflet]	Version 3.0	01 September 2016
Research protocol or project proposal [Mephedrone administration	Version 1.0	14 February 2016

protocol]		
Summary CV for student [Joanna Czerwinska CV]		18 August 2016
Summary CV for supervisor (student research) [Paul Dargan CV]		23 August 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at
http://www.hra.nhs.uk/hra-training/

16/LO/1342	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Pp

Dr Margaret Jones

Chair

Email: nrescommittee.london-riverside@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Elizabeth Bruna*
Jennifer Boston, Guy's & St Thomas' Foundation NHS Trust

APPENDIX B

Collection of drug-free matrices - ethical approval letter

Research Ethics
Office

Franklin Wilkins Building
5.9 Waterloo Bridge Wing
Waterloo Road
London SE1 9NH
Telephone 020 7848 4020/4070/4077
rec@kcl.ac.uk

KING'S College LONDON

Mark Parkin

11 April 2017

Dear Mark,

Study Title: Collection of biological samples for developing drug detection assays

Study Reference: HR-16/17-4237

I am pleased to inform you that full approval for your project has been granted by the BDM Research Ethics Subcommittee .

For your information, ethical approval has been granted for 3 years from 11 April 2017. If you need approval beyond this point, you will need to apply for an extension at least two weeks before this. You will be required to explain the reasons for the extension. However, you will not need to submit a full re-application unless the protocol has changed.

Ethical approval is required to cover the data-collection phase of the study. This will be until the date specified in this letter. However, you do not need ethical approval to cover subsequent data analysis or publication of the results. For secondary data-analysis, ethical approval is applicable to the data that is sensitive or identifies participants.

Please ensure that you follow the guidelines for good research practice as laid out in UKRIO's Code of Practice for research:
http://www.kcl.ac.uk/innovation/research/support/conduct/cop/index.aspx

Please note you are required to adhere to all research data/records management and storage procedures agreed to as part of your application. This will be expected even after the completion of the study.

If you do not start the project within three months of this letter, please contact the Research Ethics Office.

Please note that you will be required to obtain approval to modify the study. This also encompasses extensions to periods of approval. Please refer to the URL below for further guidance about the process:

http://www.kcl.ac.uk/innovation/research/support/ethics/applications/modifications.aspx

Please would you also note that we may, for the purposes of audit, contact you from time to time to ascertain the status of your research.

If you have any query about any aspect of this ethical approval, please contact the Research Ethics Office:

(http://www.kcl.ac.uk/innovation/research/support/ethics/contact.aspx)

We wish you every success with this work.

Yours sincerely,

Senior Research Ethics Officer

For and on behalf of

Chair of the BDM Research Ethics Subcommittee

Cc: Supervisor

APPENDIX C

Stability of mephedrone and five of its Phase I metabolites in human whole blood

Czerwinska J, Parkin MC, Dargan PI, George C, Kicman AT, Abbate V. Stability of mephedrone and five of its phase I metabolites in human whole blood. *Drug Test Anal.* 2018;11(4):586-594.

Stability of mephedrone and five of its phase I metabolites in human whole blood

J. Czerwinska [a], M. C. Parkin [a, d], P. I. Dargan [b, c], C. George [e], A. T. Kicman [a], V. Abbate [a, *]

[a] Analytical, Environmental and Forensic Sciences, King's College London, London, UK

[b] Clinical Toxicology, Guy's and St Thomas' NHS Foundation Trust and King's Health Partners, London, UK

[c] Clinical Toxicology, Faculty of Life Sciences and Medicine, King's College London, London, UK

[d] Now at Eurofins Forensic Services, Teddington, UK

[e] Alere Toxicology (now part of Abbott), UK

*Correspondence to: Dr Vincenzo Abbate, King's College London, Analytical, Environmental and Forensic Sciences, 150 Stamford Street, London, SE1 9NH, UK

E-mail: vincenzo.abbate@kcl.ac.uk

Keywords: mephedrone; metabolites; whole blood; stability; LC-MS/MS

DOI: 10.1002/dta.2525

1 Abstract

Mephedrone is a new psychoactive substance known to be unstable in biological matrices stored at room temperature or refrigerated. While the instability of mephedrone has been investigated before, there is currently no data regarding the stability of mephedrone metabolites. In this study, a liquid chromatography tandem mass spectrometry method for the quantification of mephedrone and five of its phase I metabolites (dihydro-mephedrone, nor-mephedrone, hydroxytolyl-mephedrone, 4-carboxy-mephedrone and dihydro-nor-mephedrone) in human whole blood has been developed and validated. Samples were extracted by a mixed mode solid phase extraction and analyzed on a pentafluorophenylpropyl column. The method was successfully validated for selectivity, linearity (0.2-2 to 10-100 ng/mL), limits of detection (50-500 pg/mL) and quantification (200-2000 pg/mL), precision (0.924-8.27%), accuracy (86.6-115%), carryover, recovery (32.5-88.3%) and matrix effects (71.0-108%). Analyte stability in human whole blood preserved with sodium fluoride/potassium oxalate was assessed at +4°C and -20°C after 24 h, 48 h, 4 days and 10 days of storage. Instability was observed in samples stored at +4°C with nor-mephedrone and 4-carboxy-mephedrone losing 40.2 ± 6.7% and 48.1 ± 4.8%, respectively, of their initial concentration at low concentration level and 33.8 ± 4.2% and 44.6 ± 6.5%, respectively, at high concentration level after 10 days. All analytes were more stable at -20°C where the highest loss of 22.6 ± 6.9% was observed for 4-carboxy-mephedrone after 10 days. This is the first time stability of mephedrone metabolites in human whole blood has been assessed, indicating -20°C to be the recommended storage condition for all analytes in clinical settings.

2 Introduction

Mephedrone (4-methylmethcathinone) is a synthetic cathinone derivative which has a similar chemical structure and desired/adverse effects to other stimulant recreational drugs such as amphetamine [1,2]. Mephedrone was first introduced to the United Kingdom (UK) recreational drug market in 2007/8 and over the last few years it has established itself as a widely used new psychoactive substance, responsible for significant morbidity and mortality [2,3].

Despite being classified in April 2010 as a Class B drug under the UK Misuse of Drugs Act of 1971 there is some evidence suggesting that mephedrone use in the UK and in particular in London remains popular [4]. Mephedrone was detected in 1.0% (n=34) of the death cases analyzed by the Toxicology Unit at Imperial College London in 2014 and this number increased to 1.5% (n=22) in 2015 [4]. There is also evidence describing an increasing problem with people injecting mephedrone which leads to higher rates of hepatitis C, HIV and acute toxicity [5].

Studies investigating the metabolism of mephedrone have been performed *in vitro* [6,7] and *in vivo*, both in animal (rat) models [8] and in humans [9]. The main phase I metabolic pathways include N-demethylation of the secondary amine to yield nor-mephedrone (NOR), reduction of the ketone moiety to the hydroxyl group to produce dihydro-mephedrone (DHM) and oxidation of the tolyl moiety, leading to the formation of hydroxytolyl-mephedrone (HYDROXY) and 4-carboxy-mephedrone (4-CARBOXY). A simultaneous reduction of the ketone moiety and N-demethylation of the secondary amine produces dihydro-nor-mephedrone (DHNM). Hepatic CYP2D6 was found to be the main enzyme responsible for the metabolism of mephedrone in humans, with only a negligible contribution from other CYP enzymes [6].

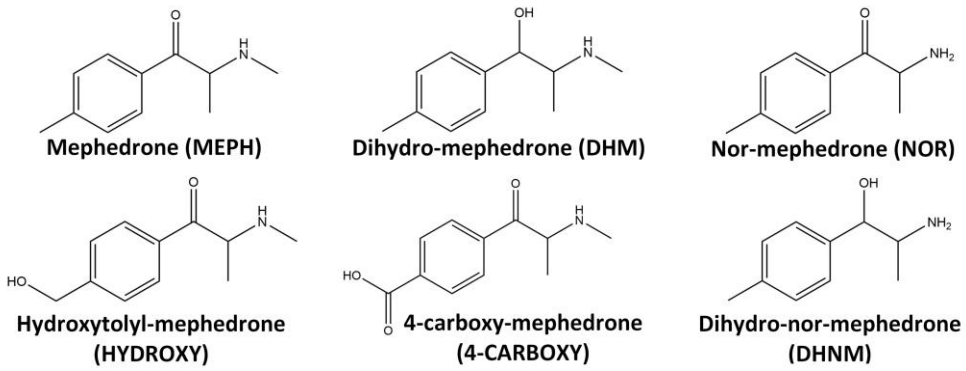

Figure 1. *Mephedrone and five of its phase I metabolites*

The stability of mephedrone has previously been investigated in human whole blood containing different preservatives and stored under different conditions [10–13]. Mephedrone has been reported to be most stable at -20°C when preserved with acidic preservatives (NaF/KOx and NaF/citrate buffer). The underlying cause of its instability in biological matrices is unknown but the previous study looking at mephedrone degradation in alkaline solution suggests the involvement of oxidants such as dissolved oxygen [14].

In this study, we investigated the stability of mephedrone and five of its phase I metabolites (Figure 1) in human whole blood fortified with NaF/ KOx as a preservative and anti-coagulant, respectively. A systematic stability study of the main mephedrone metabolites in whole blood has not been reported previously and therefore, this is an important investigation relevant to both clinical and forensic toxicologists.

3 Materials and methods

3.1 Reagents

Mephedrone hydrochloride (MEPH), dihydro-mephedrone hydrochloride (DHM), mephedrone-d_3 hydrochloride (MEPH-d_3), dihydro-mephedrone-d_3 hydrochloride (DHM-d_3), 4-(2-aminoethyl) benzoic acid hydrochloride (AEBA) and sodium borohydride were purchased from Sigma-Aldrich (Dorset, UK). Nor-mephedrone hydrochloride (NOR) was purchased from Chiron (Trondheim, Norway). Hydroxytolyl-mephedrone hydrochloride (HYDROXY), 4-carboxy-mephedrone hydrochloride (4-CARBOXY) as well as nor-mephedrone hydrochloride used for the in-house synthesis of dihydro-nor-mephedrone (DHNM) were purchased from LGC Standards (Bury, UK). MEPH, MEPH-d_3, DHM, DHM-d_3 were purchased as certified reference materials. All reference standards were analyzed in-house to verify their chemical structure.

All solvents were HPLC grade unless stated otherwise. Methanol (MeOH), isopropyl alcohol (IPA), dichloromethane (DCM), acetonitrile (LC-MS grade for the preparation of the mobile phase and HPLC grade for other uses), formic acid, acetic acid, sodium phosphate monobase, sodium phosphate diabase and ammonium hydroxide (0.88, 35%) were purchased from Fisher Scientific (Loughborough, UK). Ultrapure water (18 MΩcm) was prepared on an ELGA Purelab Maxima HPLC water purification system (High Wycombe, UK). Xtrackt® DAU High Flow (150 mg, 3 mL) cartridges were purchased from Chromatography Direct (Runcorn, UK).

Drug-free whole blood (5 mL) was collected by trained phlebotomists into vacutainers containing NaF/KOx (0.25% w/v/0.1% w/v). Ethical approval for the collection of drug-free matrix was granted by the Research Ethics Committee at King's College London (HR-16/17-4237).

3.2 Synthesis of dihydro-nor-mephedrone (DHNM)

Ten mg of NOR (61.3 µmol) was reduced to DHNM following a method described elsewhere [15]. Synthesized product was stored at -40°C and a small amount was characterized using high resolution mass spectrometry to determine its accurate mass (ThermoFisher Scientific Q-Exactive operated in positive electrospray ionization mode). Nuclear magnetic resonance (NMR) was also performed on a Bruker Avance DRX 400 MHz instrument.

3.3 Working solutions

Working solutions used for the preparation of the calibration curve were made in MeOH:water (50:50 v/v) at 4, 8, 16, 20, 100, 160, 200 ng/mL for MEPH, DHM, NOR, DHNM; 4, 10, 20, 100, 200, 400, 500 ng/mL for HYDROXY; and 40, 100, 200, 500, 1000, 1600, 2000 ng/mL for 4-CARBOXY. Working solution used for the preparation of the quality control samples at low, medium and high level were made in MeOH:water (50:50 v/v) at 5, 20, 160 ng/mL for MEPH, DHM, NOR, DHNM; 5, 40, 400 ng/mL for HYDROXY; and 50, 400, 1600 ng/mL for 4-CARBOXY. Internal standard (IS) solution containing MEPH-d_3, DHM-d_3 at 50 ng/mL and AEBA at 500 ng/mL was prepared in MeOH:water (50:50 v/v).

3.4 Calibration standards (STD) and quality control (QC) samples

Matrix-matched calibration standards containing MEPH, DHM, NOR, DHNM at 0, 0.2, 0.4, 0.8, 1, 5, 8 and 10 ng/mL; HYDROXY at 0, 0.2, 0.5, 1, 5, 10, 20 and 25 ng/mL; and 4-CARBOXY at 0, 2, 5, 10, 25, 50, 80 and 100 ng/mL were prepared by the addition of an appropriate volume of the working solution to whole blood. QC Low (0.250 ng/mL for MEPH, DHM, NOR, DHNM, HYDROXY; and 2.5 ng/mL for 4-CARBOXY), Medium (1 ng/mL for MEPH, DHM, NOR, DHNM; 2 ng/mL for HYDROXY; and 20 ng/mL for 4-CARBOXY) and High (8 ng/mL for MEPH, DHM, NOR, DHNM; 20 ng/mL for HYDROXY; and 80 ng/mL for 4-CARBOXY) were prepared by the addition of an appropriate volume of the working solution to whole blood. Calibration standards and QCs were prepared fresh on the day of sample analysis.

3.5 Sample analysis

One hundred µL of whole blood (NaF/KOx) was extracted using solid phase extraction (SPE). Ten µL of IS or 10 µL of MeOH:water (50:50 v/v) was added to the samples and solvent/matrix blanks, respectively. All samples were vortex mixed and 1 mL of 0.1 M phosphate buffer (pH 6.0) was added. After conditioning the SPE cartridges (mixed mode cation exchange containing C8 and benzoylsulfonate anion) with 2 mL of MeOH and 2 mL of 0.1 M phosphate buffer (pH 6.0), samples were loaded and washed with 2 mL of 0.1 M acetic $acid_{(aq)}$ followed by 2 mL of MeOH. Samples were eluted with 4 x 1 mL of DCM:IPA:ammonium hydroxide (78:20:2 v/v/v) and dried under nitrogen at 50°C. Samples were reconstituted with 100 µL of 0.1% formic acid in ACN:water (10:90 v/v).

3.6 LC-MS/MS conditions

The analysis was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Waters Xevo TQ-S triple quadrupole mass spectrometer (Manchester, UK) coupled to Waters Acquity ultra performance liquid chromatograph system equipped with a CTC 2777 open architecture autosampler (Waters, UK).

Extracted samples were analyzed using electrospray ionization operated in positive ion mode. The source temperature was set at 150°C. The desolvation gas flow rate was 1000 L/h at a temperature of 500°C, capillary voltage was set to 2.22 kV, cone voltage was 45 V and source offset was 84 V. The cone gas flow rate was set to 150 L/h, the nebulizer gas flow was 7.00 bar and the collision gas flow rate was 0.25 mL/min. Mephedrone metabolites and deuterated internal standards were monitored using selected reaction monitoring (SRM) as detailed in Table 1. In order to maximize sensitivity, all analytes except for 4-CARBOXY and HYDROXY had their dehydration products chosen as target precursor ions due to significant in-source fragmentation which is commonly observed in synthetic cathinones [9,16].

Chromatographic separation was performed using a 2.1 mm x 150 mm, 1.8 µm, pentafluorophenylpropyl Selectra® column (Bristol, US) held at 60°C. The strong needle wash was 0.3% formic acid in MeOH and the weak needle wash was 0.01% formic acid in acetonitrile:water (10:90 v/v). The flow rate was 0.5 mL/min with 0.3% formic acid in water as mobile phase A and 0.3% formic acid in acetonitrile as mobile phase B. The start of the gradient was at 85% mobile phase A. Mobile phase B was then increased to 55% over 11 min and was held for 2 min. Over the next 0.5 min the gradient returned to the starting condition and the column was re-equilibrated at 85% mobile phase A for the remaining 1.5 min. The total run time was 15 min. The injection volume was 20 µL and the data was acquired using MassLynx (version 4.1) software. TargetLynx (version 4.1) was used for data processing and quantitation.

Table 1*. The retention time, SRM transitions and collision energy for each ion; * denotes a quantifying transition*

Analyte	**Retention time** (min)	**Precursor ion** (*m/z*)	**Product ion** (*m/z*)	**Collision energy** (V)	**Internal standard**
MEPH	5.85	160.4	145.1*	15	MEPH-d_3
			144.1	33	
			91.1	28	
MEPH-d_3	5.85	163.4	148.4	19	
DHM	5.38	162.4	147.3*	19	DHM-d_3
			131.4	17	
			91.3	26	
DHM-d_3	5.38	165.4	150.3	18	
NOR	5.00	146.0	131.1	25	MEPH-d_3
			130.1*	25	
			119.0	15	
HYDROXY	1.98	194.1	158.1	17	DHM-d_3
			146.0*	17	

			131.1	23	
4-CARBOXY	2.06	208.0	146.0*	13	AEBA
			144.1	28	
			130.1	31	
DHNM	4.45	148.1	131.1*	13	MEPH-d_3
			116.2	23	
			91.1	25	
AEBA	1.77	166.1	149.1	10	

3.7 Stability

Stability samples prepared at QC Low and QC High levels in drug-free human whole blood (NaF/KOx) were aliquoted into tubes and stored at +4°C and -20°C for 24 h, 48 h, 4 days and 10 days. At each sampling point, one tube at each QC level was removed and six aliquots extracted. Freezer and fridge temperatures were monitored and logged daily.

4 Method validation

Validation experiments determined selectivity, linearity, inter- and intra-day precision and accuracy, limit of detection (LOD) and limit of quantification (LOQ), recovery, matrix effect, carryover and stability according to the validation guidelines published by the Food and Drug Administration (FDA) [17] and recommendations published by Peters at el. [18]

4.1 Selectivity

Selectivity was assessed by analyzing 6 blank whole blood samples collected from 3 drug-free female and 3 drug-free male donors.

4.2 Linearity

Matrix-matched calibration curve was prepared by fortifying drug-free whole blood (NaF/KOx) with appropriate working solutions containing mephedrone and its metabolites. Each calibration standard was required to be within ± 20% of its target concentration and the correlation coefficient (r^2) of the line to be at least 0.99. A linear regression model with a weighting of 1/x was applied to the calibration curve.

4.3 LOD and LOQ

The LOD for each analyte was defined as the lowest concentration where all 3 ions (2 qualifier and 1 quantifier) were present with a signal-to-noise ratio equal to or greater than 3. The LOQ was defined as the lowest concentration at which analytes could be quantified with an acceptable precision and accuracy (± 20%). The upper limit of quantification was determined as the highest concentration of the calibration line, which could be determined with an acceptable accuracy and precision (± 20%) without saturating the instrument signal.

4.4 Precision and accuracy

Intra-day (n=6) and inter-day (n=3) precision and accuracy was determined by employing QC samples spiked at low, medium and high concentrations. Intra-day precision was calculated using 6 replicates obtained on the same day and expressed as a percentage relative standard deviation (% RSD). Accuracy was calculated by dividing the mean measured concentration at each QC level by the theoretical spiked concentration and expressed as a percentage of the theoretical spiked concentration. Inter-day precision was evaluated for each QC level run on three different days and expressed as % RSD. Values of ± 20% are acceptable according to the guidelines.

4.5 Recovery and matrix effect

For recovery, a set of whole blood samples (n=6) was fortified at QC Low and QC High level and was taken through the SPE. In parallel, a set of blank whole blood samples (n=6) was extracted and fortified after the evaporation step at QC Low and QC High level. Recovery was calculated as a percentage by comparing the absolute peak areas of the samples spiked before extraction with samples spiked after extraction.

For the IS-corrected matrix effect, a set of blank whole blood samples (n=6, from 3 female and 3 male individuals) and a set of water samples (n=6) was taken through the extraction. All samples were reconstituted with a solution containing known amounts of the internal standard and analytes at QC Low and QC High levels. Matrix effect was evaluated by comparing peak area ratios in blank whole blood samples fortified after extraction with peak area ratios in water samples fortified after extraction.

4.6 Carryover

Carryover was assessed by injecting methanol blanks after the highest calibration standard.

5 Results and Discussion

5.1 DHNM synthesis

DHNM was successfully synthesized (yield: 51%). Formula $C_{10}H_{16}NO^+$; HRMS [M+H$^+$] calculated m/z 166.1226, observed 166.1227 (+0.001 ppm); observed MS/MS fragments with collision energy 20 eV were consistent with those reported in the literature [9]. ^{1}H NMR ($CDCl_3$): δ 7.22 (d, J=8.0 Hz, 2H, Ar-H), 7.16 (d, J=8.0 Hz, 2H, Ar-H), 4.50 (d, J=4.0 Hz, 1H, C<u>H</u>(OH)), 3.19 (br , 1H, C<u>H</u>(CH_3)), 2.35 (s, 3H, Ar-CH_3) and 0.98 (d, J=8.0 Hz, 3H, CH(C<u>H</u>$_3$)). ^{1}H NMR data is consistent with the literature except for the signal at 3.19 ppm being previously reported as a multiplet [15].

5.2 Method validation

Selectivity

No interferences were observed in the extracted blank matrix.

Linearity

Mean linearity of $r^2 > 0.998$ was achieved for each analyte in all 3 validation runs.

LOD and LOQ

LOD and LOQ of 50 pg/mL and 200 pg/mL, respectively, was achieved for all analytes except 4-CARBOXY for which LOD and LOQ was 500 pg/mL and 2000 pg/mL, respectively. Table 2 shows calibration parameters for all analytes.

Table 2. *LOD, LOQ, calibration range and calibration parameters for all analytes*

Analyte	**LOD** (pg/mL)	**LOQ** (pg/mL)	**Range** (ng/mL)	**Intercept ± SD** (n=3)	**Slope ± SD** (n=3)	**r^2 ± SD** (n=3)
MEPH	50	200	0.2-10	-0.0127 ± 0.0263	3.15 ± 0.1290	0.999 ± 0.0000
DHM	50	200	0.2-10	-0.0324 ± 0.0043	2.81 ± 0.0529	0.998 ± 0.0010
NOR	50	200	0.2-10	-0.0147 ± 0.0088	1.65 ± 0.0608	0.997 ± 0.0000
HYDROXY	50	200	0.2-25	-0.000510 ± 0.0096	1.21 ± 0.1300	0.998 ± 0.0010
4-CARBOXY	500	2000	2-100	6.85 ± 5.8800	27.9 ± 23.6000	0.997 ± 0.0015
DHNM	50	200	0.2-10	-0.00381 ± 0.0073	2.10 ± 0.2000	0.998 ± 0.0006

Precision and accuracy

Intra-day and inter-day precision and accuracy results, summarized in Table 3**,** were found to be within the acceptable limits. The intra-day accuracy for all metabolites was within ± 15% of the target concentration and ranged from 96.7-106% for MEPH, 91.1-109% for DHM, 89.7-97.0% for NOR, 94.3-115% for HYDROXY, 97.0-114% for 4-CARBOXY and 86.6-103% for DHNM. The intra-day precision was < 7% and ranged from 1.44-4.33% for MEPH, 0.924-4.65% for DHM, 1.58-4.87% for NOR, 1.55-6.57% for HYDROXY, 1.36-5.97% for 4-CARBOXY, 1.52-5.13% for DHNM. Inter-day precision and accuracy results were acceptable over the validated range with % RSD < 8.5% and accuracy within ± 9.0% of the target concentration.

Table 3*. Precision and accuracy at QC Low, QC Medium and QC High*

Analyte	**True value** (ng/mL)	**Mean** (ng/mL), **(% RSD)**, **% accuracy**			
		Day 1 n=6	Day 2 n=6	Day 3 n=6	Inter-day n=18
MEPH	0.250	0.246 (4.33%) 98.2%	0.251 (1.44%) 101%	0.248 (3.45%) 99.3%	0.248 (3.24%) 99.3%
	1	1.00 (2.92%) 100%	0.967 (2.33%) 96.7%	1.00 (1.88%) 100%	0.988 (2.77%) 98.8%
	8	8.47 (1.72%) 106%	7.92 (2.40%) 99.0%	7.88 (1.48%) 98.6%	8.08 (3.84%) 101%
DHM	0.250	0.273 (2.68%) 109%	0.228 (2.80%) 91.1%	0.256 (4.65%) 102%	0.252 (8.27%) 101%
	1	1.02 (4.06%) 102%	0.992 (1.74%) 99.2%	1.04 (2.98%) 104%	1.02 (3.53%) 102%
	8	8.15 (0.924%) 102%	7.84 (2.22%) 98.0%	7.79 (4.05%) 97.3%	7.91 (3.28%) 98.9%
NOR	0.250	0.229 (2.55%) 91.6%	0.238 (2.97%) 95.1%	0.231 (4.87%) 92.5%	0.233 (3.77%) 93.1%
	1	0.933 (3.01%) 93.3%	0.928 (3.63%) 92.8%	0.932 (1.58%) 93.2%	0.931 (2.70%) 93.1%
	8	7.76 (3.87%) 97.0%	7.40 (4.56%) 92.5%	7.18 (3.63%) 89.7%	7.44 (5.03%) 93.0%
HYDROXY	0.250	0.236 (5.53%) 94.3%	0.267 (4.70%) 107%	0.253 (2.72%) 101%	0.252 (6.69%) 101%
	2	2.07	2.29	2.14	2.17

		(3.49%)	(3.89%)	(2.37%)	(5.42%)
		103%	115%	107%	108%
		21.1	23.0	20.1	21.4
	20	(6.57%)	(4.75%)	(1.55%)	(7.38%)
		105%	115%	100%	107%
		2.42	2.48	2.43	2.44
	2.5	(1.36%)	(3.93%)	(4.26%)	(3.48%)
		97.0%	99.1%	97.0%	97.7%
		20.7	21.9	22.9	21.8
4-CARBOXY	20	(5.44%)	(5.97%)	(3.95%)	(6.40%)
		103%	109%	114%	109%
		85.2	85.3	87.6	86.1
	80	(5.40%)	(3.37%)	(3.60%)	(4.21%)
		107%	107%	109%	108%
		0.228	0.238	0.239	0.235
	0.250	(3.46%)	(4.55%)	(1.52%)	(3.78%)
		91.3%	95.0%	95.4%	93.9%
		0.970	0.922	0.937	0.943
DHNM	1	(5.13%)	(3.72%)	(4.99%)	(4.92%)
		97.0%	92.2%	93.7%	94.3%
		8.22	7.49	6.93	7.55
	8	(4.00%)	(4.01%)	(2.21%)	(7.97%)
		103%	93.6%	86.6%	94.3%

Recovery and matrix effect

Recovery was found to be greater than 71.3% for all analytes, except for 4-CARBOXY for which recovery was 32.5 ± 6.8% at QC Low level and 41.6 ± 0.5% at QC High level. 4-CARBOXY is a zwitterionic compound which contains an acidic carboxylic acid group and a basic secondary amine group. According to Marvin (chemistry software package, version 17.16.0), the secondary amine has a pKa of 8.0 and the carboxylic acid has a pKa of 3.6, similar to the pKa values of mephedrone and benzoic acid, respectively. Secondary amines become fully protonated upon the addition of diluted acetic acid (pH 2.9) during the wash step in SPE, whereas only about 80% of the carboxylic acid group is protonated under this pH. Therefore, about 20% of the molecule exists as a neutral zwitterion with no net charge. A subsequent wash with 2 mL of MeOH to remove neutral and acidic interferents, such as free fatty acids, will also disrupt the hydrophobic interaction of this 'net neutral' metabolite with the C8 alkyl chains, causing a considerable proportion to be lost which might explain lower recovery for 4-CARBOXY. The other analytes are all basic and as cations will ionically interact with the benzoylsulfonate anion within the mixed-mode stationary phase during the MeOH wash, which results in higher recovery. Even though it is recommended for the recovery to be greater than 50% [18], desired sensitivity as well as acceptable precision and accuracy were achieved for 4-CARBOXY (Table 4).

IS-corrected matrix effect values were within ± 17% at both QC Low and QC High level, except for HYDROXY at QC High which was suppressed by 29.0% (Table 4). This may be due to the lack of matching deuterated IS which is currently not commercially available. However, assay precision and accuracy for HYDROXY at QC High was within the acceptable limits (Table 3).

Table 4. *Analyte recovery and matrix effect at QC Low and QC High*

Analyte	**Recovery (% RSD)**, n=6		**Matrix Effect (% RSD)**, n=6	
	QC LOW	QC HIGH	QC LOW	QC HIGH
MEPH	85.2% (1.84%)	88.3% (3.17%)	101% (8.16%)	99.0% (1.12%)
DHM	83.6% (9.97%)	84.2% (2.70%)	105% (2.87%)	98.7% (0.792%)
NOR	74.3% (3.78%)	76.6% (3.31%)	89.6% (5.89%)	91.7 % (3.25%)
HYDROXY	71.3% (4.12%)	81.4% (2.62%)	83.8% (2.58%)	71.0% (6.00%)
4-CARBOXY	32.5% (6.79%)	41.6% (0.522%)	103% (6.49%)	108% (5.99%)
DHNM	78.6% (5.70%)	79.0% (5.41%)	93.0% (4.66%)	87.4% (4.94%)

Carryover

Carryover was not observed.

5.3 Stability

Stability data together with corresponding % RSD is presented in Table 5 and showed graphically in Figures 2-5. Analytes were considered unstable when they lost more than 10% of their initial concentration.

At +4°C at QC Low, DHM and DHNM were stable over the 10-day period while HYDROXY and MEPH lost 18.6 ± 5.2% and 23.4 ± 6.3%, respectively, of their initial concentration. 4-CARBOXY and NOR decreased in concentration by 48.1 ± 4.8% and 40.2 ± 6.7%, respectively, after 10 days (Figure 2). At QC High, DHM and DHNM were stable over the 10-day period while HYDROXY and MEPH lost 11.3 ± 3.2% and 14.2 ± 3.3%, respectively, of their initial concentration. 4-CARBOXY and NOR were most unstable and their concentration decreased by 44.6 ± 6.5% and 33.8 ± 4.2%, respectively, after 10 days (Figure 3).

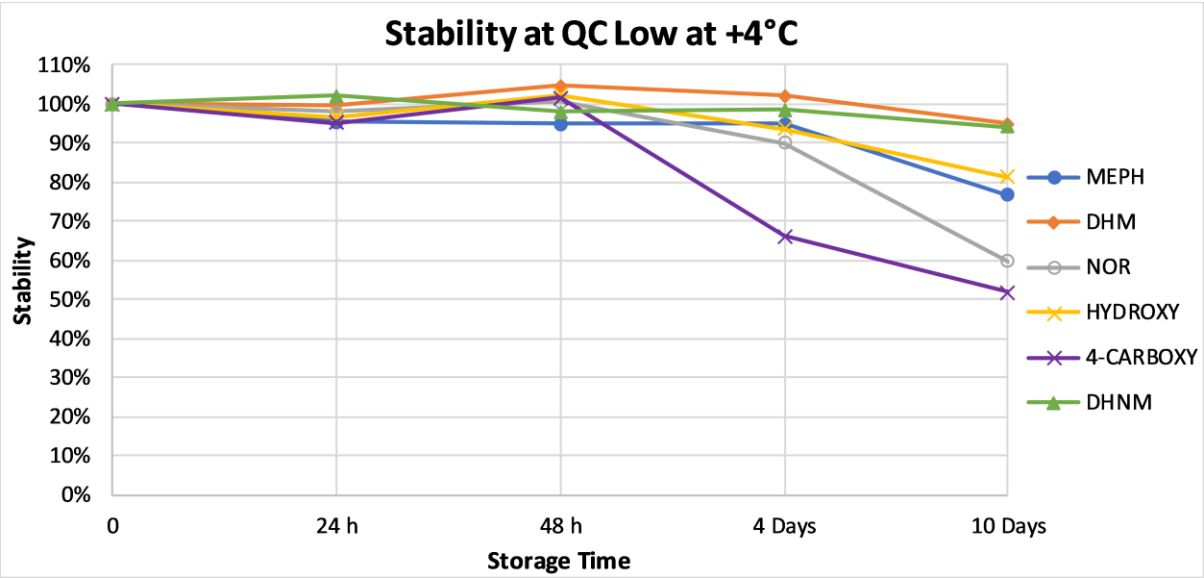

Figure 2. *Analyte stability at QC Low at +4°C*

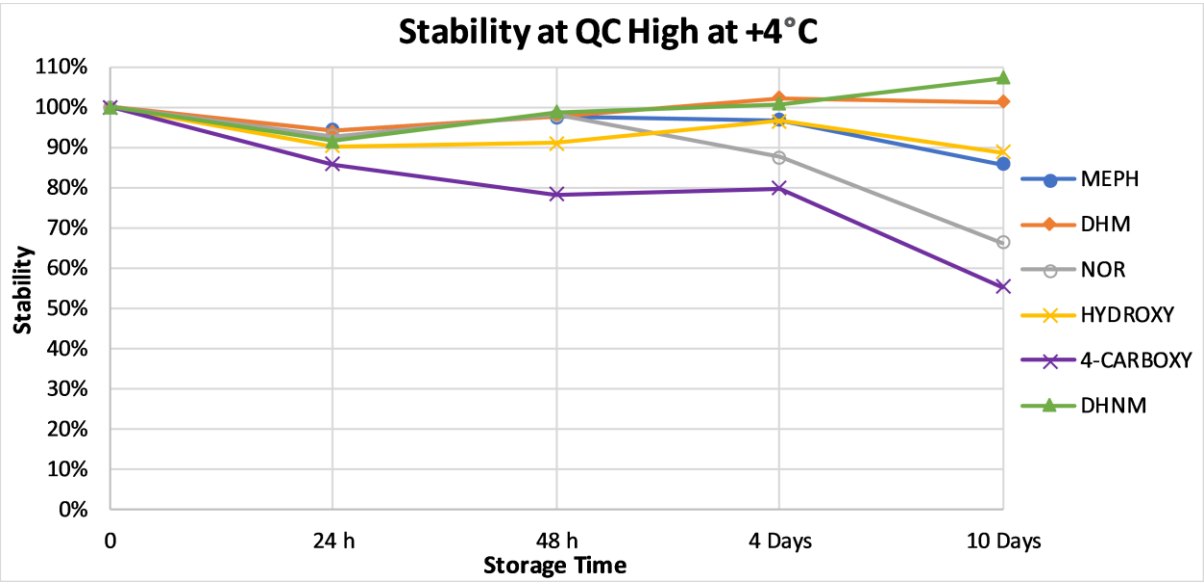

Figure 3. *Analyte stability at QC High at +4°C*

At -20°C at QC Low, NOR and DHNM were most stable over the 10-day period while MEPH, 4-CARBOXY, HYDROXY and DHM lost 9.9 ± 2.4%, 9.6 ± 5.3%, 11.2 ± 4.8% and 12.0 ± 4.8%, respectively, of their initial concentration (Figure 4). At QC High, 4-CARBOXY was the most unstable and decreased in concentration by 22.6 ± 6.9% after 10 days. MEPH, DHM, NOR and DHNM were stable over the 10-day period while HYDROXY lost 10.2 ± 2.2% of its initial concentration (Figure 5).

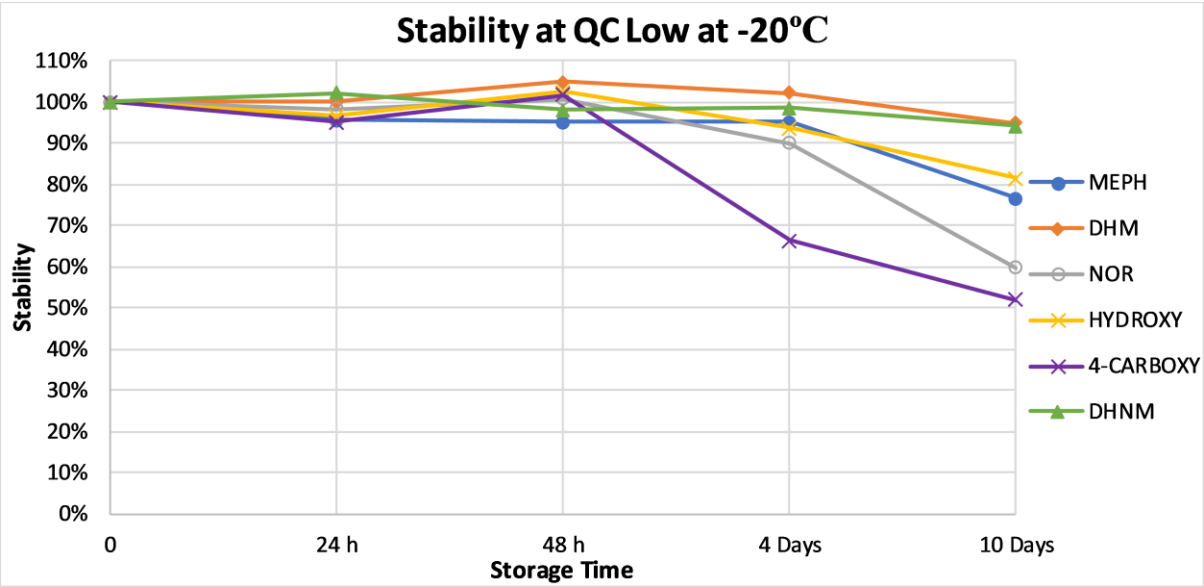

Figure 4. *Analyte stability at QC Low at -20°C*

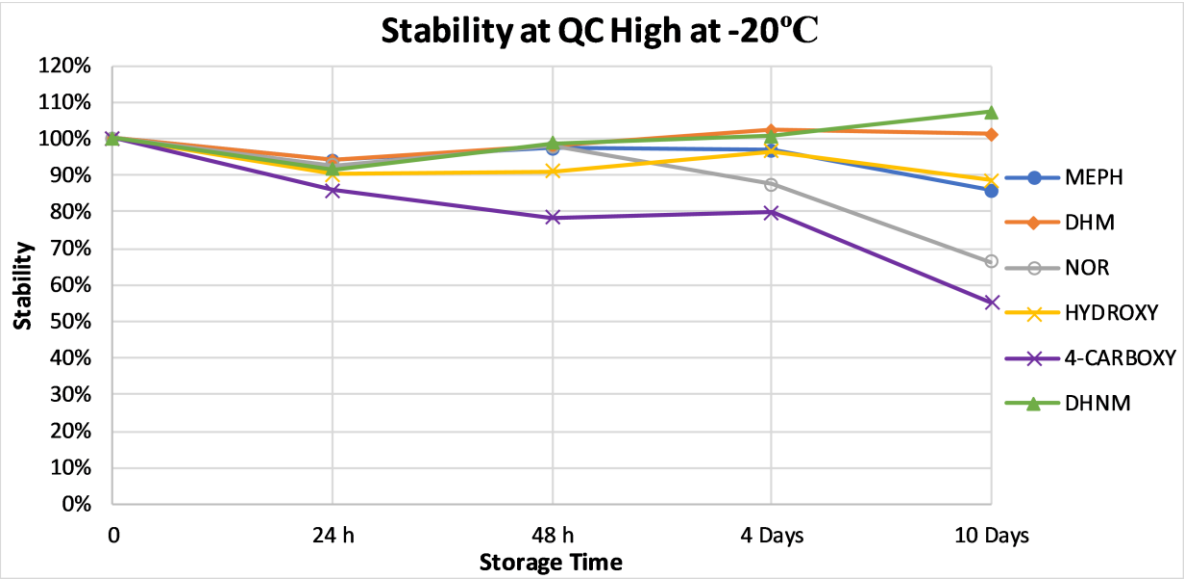

Figure 5. *Analyte stability at QC High at -20°C*

Out of all metabolites, 4-CARBOXY was the most unstable at +4°C with significant losses observed already after 4 days (33.7 ± 6.1%) at QC Low and after 48 h (21.6 ± 4.3%) at QC High. Its stability was improved at -20°C where the highest loss of 22.6 ± 6.9% was observed after 10 days at QC High. NOR was much more stable at -20°C than 4°C where it lost 40.2 ± 6.7% at QC Low (versus no change at -20°C) and 33.8 ± 4.2% at QC High (versus 6.6 ± 3.6% at -20°C). HYDROXY was stable at -20°C but lost 18.6 ± 5.2% at +4°C after 10 days at QC Low. DHM and DHNM were most stable at +4°C at both concentration levels with the latter showing a slight increase in its concentration after 10 days at QC High. DHM and DHNM are the only two

metabolites containing a hydroxyl group instead of a ketone at the β carbon which was previously reported to make ephedrines more stable than cathinones [11].

Table 5. *QC Low and QC High concentrations ± % RSD (% loss/gain) for each analyte after 24 h, 48 h, 4 days and 10 days of storage at +4°C and -20°C*

Analyte	QC level, conc. (ng/mL)	24 h		48 h		4 Days		10 Days	
		+4°C	-20°C	+4°C	-20°C	+4°C	-20°C	+4°C	-20°C
MEPH	QC Low, 0.250	0.239 ± 1.31% (-4.4%)	0.246 ± 5.46% (-1.8%)	0.238 ± 5.75% (-4.9%)	0.259 ± 0.306% (+4.0%)	0.238 ± 4.25% (-4.9%)	0.260 ± 5.79% (+4.0%)	0.192 ± 6.28% (-23.4%)	0.225 ± 2.35% (-9.9%)
	QC High, 8	7.53 ± 2.60% (-5.8%)	8.39 ± 4.07% (+5.0%)	7.79 ± 2.19% (-2.6%)	8.63 ± 3.50% (+8.0%)	7.74 ± 3.75% (-3.3%)	8.25 ± 5.05% (+3.0%)	6.86 ± 3.34% (-14.2%)	7.88 ± 2.03% (-1.5%)
DHM	QC Low, 0.250	0.250 ± 2.08% (-0.1%)	0.243 ± 4.02% (-2.8%)	0.262 ± 7.26% (+5.0%)	0.243 ± 2.74% (-2.8%)	0.256 ± 1.12% (+2.0%)	0.240 ± 3.88% (-4.0%)	0.237 ± 3.70% (-5.0%)	0.220 ± 4.83% (-12.0%)
	QC High, 8	7.52 ± 2.87% (-6.0%)	8.36 ± 4.82% (+5.0%)	7.82 ± 0.857% (-2.2%)	8.52 ± 3.93% (+7.0%)	8.17 ± 1.90% (+2.0%)	8.24 ± 5.06% (+3.0%)	8.10 ± 0.619% (1.0%)	7.79 ± 2.04% (-2.6%)
NOR	QC Low, 0.250	0.245 ± 3.97% (-1.8%)	0.248 ± 3.19% (-0.8%)	0.252 ± 5.65% (+1.0%)	0.246 ± 6.97% (-1.4%)	0.225 ± 4.81% (-10.0%)	0.255 ± 7.22% (+2.0%)	0.149 ± 6.65% (-40.2%)	0.258 ± 7.50% (+3.0%)
	QC High, 8	7.41 ± 3.14% (-7.4%)	8.36 ± 4.05% (+4.0%)	7.84 ± 2.54% (-2.0%)	8.83 ± 4.93% (+10.0%)	6.99 ± 4.04% (-12.6%)	8.04 ± 3.41% (0.0%)	5.30 ± 4.18% (-33.8%)	7.48 ± 3.63% (-6.6%)
HYDROXY	QC Low, 0.250	0.242 ± 5.57% (-3.3%)	0.235 ± 3.40% (-6.0%)	0.256 ± 10.0% (+2.4%)	0.239 ± 4.20% (-4.4%)	0.234 ± 8.81% (-6.4%)	0.221 ± 4.71% (-11.4%)	0.203 ± 5.18% (-18.6%)	0.222 ± 4.84% (-11.2%)
	QC High, 20	18.1 ± 0.933% (-9.7%)	18.8 ± 5.33% (-5.9%)	18.2 ± 4.89% (-8.9%)	19.2 ± 5.32% (-4.0%)	19.3 ± 3.56% (-3.6%)	19.3 ± 4.85% (-3.5%)	17.7 ± 3.16% (-11.3%)	18.0 ± 2.22% (-10.2%)
4-CARBOXY	QC Low,	2.38 ± 5.00%	2.49 ± 5.82%	2.54 ± 5.14%	2.56 ± 6.92%	1.66 ± 6.07%	2.61 ± 6.51%	1.30 ± 4.79%	2.26 ± 5.30%

	2.5	(-4.8%)	(-0.5%)	(+2.0%)	(+2.0%)	(-33.7%)	(+4.0%)	(-48.1%)	(-9.6%)
	QC High, 80	68.6 ± 5.38% (-14.2%)	78.6 ± 2.17% (-1.7%)	62.7 ± 4.30% (-21.6%)	87.5 ± 10.8% (+9.0%)	63.9 ± 4.61% (-20.2%)	81.8 ± 8.27% (+2.0%)	44.3 ± 6.51% (-44.6%)	61.9 ± 6.87% (-22.6%)
DHNM	QC Low, 0.250	0.255 ± 1.86% (+2.0%)	0.253 ± 0.714% (+1.0%)	0.245 ± 7.90% (-1.9%)	0.251 ± 6.47% (0.0%)	0.247 ± 5.22% (-1.4%)	0.251 ± 3.92% (0.0%)	0.236 ± 3.71% (-5.7%)	0.254 ± 4.94% (+2.0%)
	QC High, 8	7.33 ± 1.75% (-8.4%)	8.51 ± 0.724% (+6.0%)	7.90 ± 2.34% (-1.3%)	8.62 ± 1.49% (+8.0%)	8.06 ± 3.66% (+1.0%)	8.18 ± 2.85% (+2.0%)	8.57 ± 4.80% (+7.0%)	7.39 ± 3.33% (-7.6%)

Studies investigating the stability of mephedrone in human whole blood have been published before. Sørensen [11] investigated the stability of cathinones (including mephedrone) and related ephedrines in human whole blood spiked with analytes at 100 µg/mL and preserved with NaF/KOx or NaF/citrate buffer. Samples were stored at either +4°C or +20°C for up to 5 or 6 days. After 5 days of storage mephedrone was more stable at +4°C than +20°C. Busardò *et al.* [12] reported on the stability of mephedrone in ante-mortem and post-mortem blood preserved with NaF/KOx or EDTA. Whole blood samples were spiked at 1 mg/mL and stored at -20°C, +4°C or +20°C for up to 185 days. Mephedrone was shown to be most stable in ante-mortem samples at all tested storage conditions, with -20°C being the best storage temperature. This study showed that mephedrone stability is pH dependent and acidic preservatives are better suited (6.6% vs 9.4% loss after 185 days at -20°C when preserved with NaF/KOx rather than EDTA). Johnson and Botch-Jones [10] investigated the stability of four designer drugs (including mephedrone) stored at -20°C, +4°C or +22°C over 14 days. Human whole blood (preservative not stated), plasma and urine samples were spiked at 1 µg/mL. This study showed a mean 48% reduction in mephedrone concentration in whole blood kept at +4°C for 14 days. Over the same period of time mephedrone was undetected when stored at room temperature whilst there was no measurable degradation at -20°C. The most recent study looked at the stability of mephedrone and other synthetic cathinones in bovine blood fortified with NaF/KOx at 100 ng/mL (QC Low) and 1,000 ng/mL (QC High) stored at -20°C, +4°C, +20°C and +32°C. At QC Low a complete degradation of mephedrone was observed after 11 days when stored at the elevated temperature. Degradation was much slower at +4°C and -20°C where a 20% loss was observed after 55 days and 130 days, respectively [13]. These results follow the stability pattern seen in our study where mephedrone and its metabolites have been shown to be more stable at -20°C than +4°C.

Low concentrations of mephedrone and its metabolites were chosen for this degradation study to help distinguish subtle changes from initial values that may not be so easily detected if much higher initial concentrations were chosen. However, it is possible that the losses

observed were independent of the initial concentrations and, if so, the relative rates of instability will not apply in most casework where much higher concentrations are typically observed. It would be of interest in the future to investigate whether the relative losses observed are germane to higher concentrations. In the interim, when evaluating cases concerning mephedrone and its metabolites where sample storage conditions may be considered a factor, hopefully the overarching recommendations and conclusions of our investigation will be considered of value.

6 Conclusion

A fully validated method for the simultaneous quantification of mephedrone and five of its phase I metabolites in human whole blood (NaF/KOx) has been developed. Mephedrone's stability in whole blood has been previously investigated but this is the first time stability of its metabolites has been assessed, indicating -20°C to be the recommended storage condition for all analytes.

7 Acknowledgments

The authors would like to thank Biotechnology and Biological Sciences Research Council (grant number BB/M014940/1) and Alere Toxicology (now part of Abbott) for their financial support.

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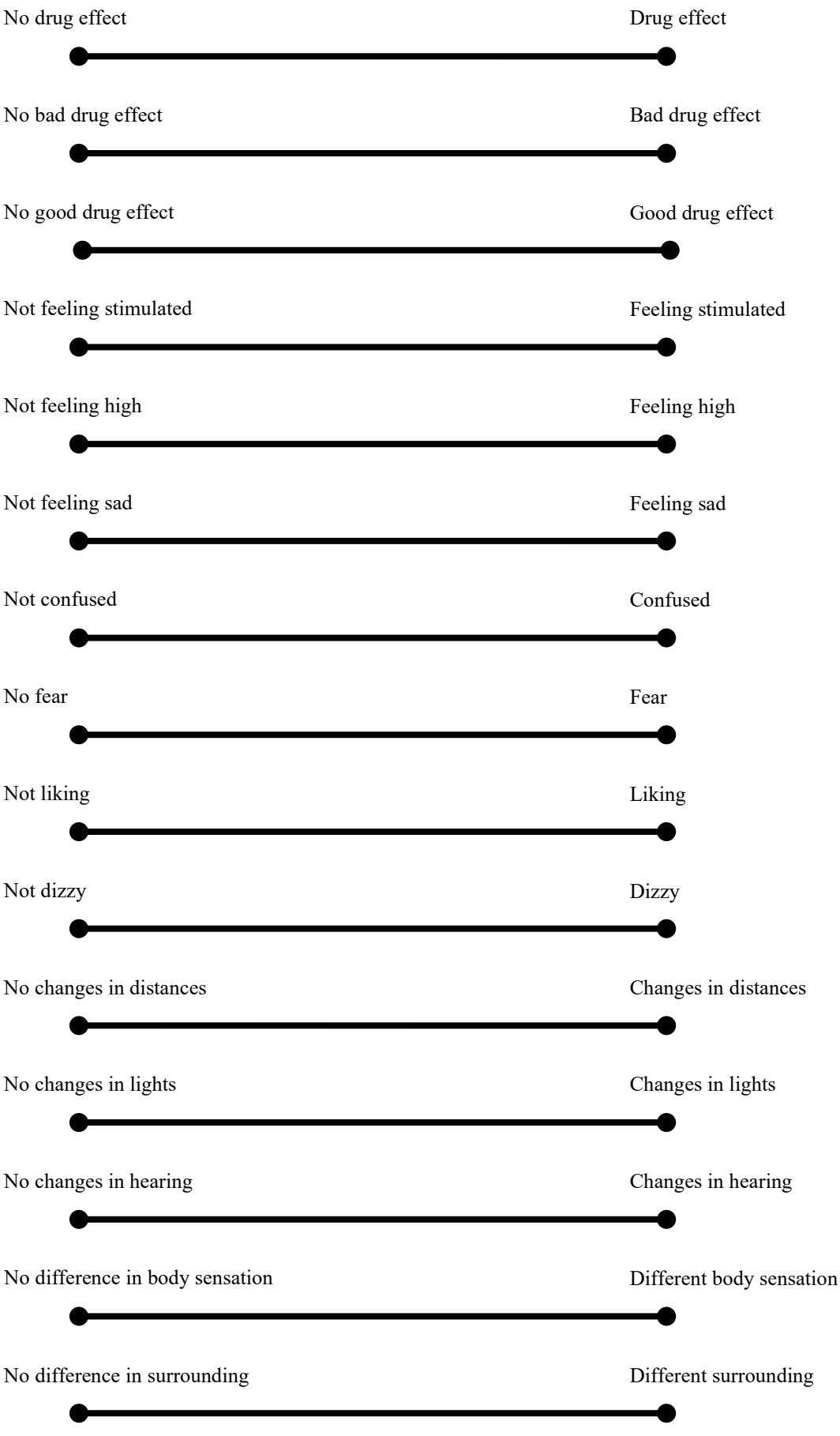
APPENDIX D

Visual analogue scale

Mark a vertical line to indicate how you feel



APPENDIX E

Individual data

1. Individual raw data showing changes in analyte concentration over time in whole blood

Table 1. Changes in **mephedrone** concentration over time in M1-M6 in whole blood;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	M2	M3	M4	M5	M6
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	49.1	26.5	81.8	34.5	61.5	179
10	63.5	41.5	78.9	48.5	71.4	158
15	63.9	47.0	74.2	54.7	85.4	150
20	64.3	70.4	68.8	63.7	86.8	140
30	65.6	76.3	67.1	74.4	108	173
45	64.3	88.2	69.7	76.7	92.8	189
60	65.0	91.1	66.8	69.9	96.2	161
75	69.1	81.9	73.8	65.5	75.6	134
90	65.7	79.7	59.4	62.5	65.9	119
105	55.2	69.1	51.2	60.4	64.0	95.1
120	55.1	65.6	47.6	52.2	57.2	99.4
150	47.7	49.0	39.4	44.5	47.7	72.4
180	43.5	37.4	34.6	35.7	39.1	67.6
300	25.9	19.6	19.3	16.3	18.3	40.4
360	20.1	16.9	14.8	13.6	13.1	29.4
Day 2	0.212	0.044	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

Table 2. Changes in **dihydro-mephedrone** concentration over time in M1-M6 in whole blood;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	M2	M3	M4	M5	M6
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	0.309	BLQ	BLQ	BLQ	0.206	0.584
10	0.407	BLQ	0.215	BLQ	0.264	0.612
15	0.434	BLQ	0.234	0.224	0.324	0.849
20	0.459	0.267	0.262	0.250	0.358	0.809
30	0.564	0.399	0.403	0.317	0.496	1.34
45	0.585	0.660	0.850	0.389	0.569	2.25
60	0.775	1.06	1.22	0.432	0.716	2.37
75	0.903	1.30	1.48	0.451	0.696	2.30
90	1.25	1.40	1.79	0.473	0.876	2.47
105	1.36	1.40	1.89	0.483	0.868	2.43

120	1.28	1.44	1.75	0.475	0.787	2.22
150	1.45	1.34	1.71	0.483	0.762	2.42
180	1.55	1.29	1.69	0.402	0.743	2.04
300	1.44	0.907	1.53	0.280	0.546	1.56
360	1.33	0.901	1.39	0.274	0.480	1.58
Day 2	BLQ	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

Table 3. Changes in **nor-mephedrone** concentration over time in M1-M6 in whole blood;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	**M2**	**M3**	**M4**	**M5**	**M6**
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
10	BLQ	BLQ	BLQ	BLQ	BLQ	0.293
15	0.219	BLQ	0.286	BLQ	BLQ	BLQ
20	0.368	0.371	0.571	0.228	0.284	0.900
30	0.894	0.980	1.14	0.644	0.706	2.32
45	1.43	2.32	2.67	1.20	1.16	5.90
60	2.33	4.04	3.75	1.45	1.86	7.13
75	3.65	5.24	4.50	1.66	2.16	7.31
90	5.49	5.76	5.57	1.85	3.00	7.75
105	6.27	5.33	5.29	1.89	3.08	6.99
120	6.08	5.78	4.44	1.97	2.84	7.15
150	6.67	5.77	4.32	2.25	2.47	7.93
180	6.50	5.68	4.05	1.96	2.47	6.90
300	5.87	3.91	3.17	1.29	1.76	5.49
360	5.34	3.74	2.87	1.25	1.61	5.10
Day 2	BLQ	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

Table 4. Changes in **hydroxytolyl-mephedrone** concentration over time in M1-M6 in whole blood;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	**M2**	**M3**	**M4**	**M5**	**M6**
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
10	BLQ	BLQ	BLQ	BLQ	BLQ	0.488
15	BLQ	0.200	BLQ	BLQ	BLQ	0.912
20	BLQ	0.312	0.289	0.203	BLQ	1.27
30	0.247	0.597	0.431	0.440	0.349	3.51
45	0.263	1.10	1.15	0.493	0.459	5.38

60	0.489	1.47	1.11	0.441	0.740	3.89
75	0.622	1.40	1.03	0.480	0.761	3.53
90	0.770	1.35	0.979	0.449	1.14	2.43
105	0.860	1.05	0.807	0.412	0.821	2.31
120	0.690	0.891	0.621	0.413	0.442	1.88
150	0.559	0.783	0.510	0.409	0.323	1.62
180	0.431	0.766	0.373	0.274	BLQ	0.989
300	0.264	0.310	BLQ	BLQ	BLQ	0.387
360	BLQ	0.213	BLQ	BLQ	BLQ	0.265
Day 2	BLQ	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

Table 5. Changes in **4-carboxy-mephedrone** concentration over time in M1-M6 in whole blood;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	**M2**	**M3**	**M4**	**M5**	**M6**
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	BLQ	14.0
10	2.83	3.17	5.81	4.57	8.20	59.2
15	13.2	19.0	11.2	15.1	23.8	62.0
20	22.0	33.4	26.4	25.3	53.2	113
30	46.4	95.6	47.3	73.3	73.2	253
45	84.6	172	103	127	119	398
60	71.2	254	106	131	170	454
75	103	194	167	143	232	352
90	116	200	166	148	253	394
105	134	175	125	158	244	381
120	162	150	119	146	215	361
150	103	131	79.1	138	153	242
180	106	107	61.2	102	109	212
300	53.5	39.6	23.8	31.0	62.7	103
360	46.2	33.2	20.4	24.7	65.1	62.4
Day 2	BLQ	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

Table 6. Changes in **dihydro-nor-mephedrone** concentration over time in M1-M6 in whole blood;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	**M2**	**M3**	**M4**	**M5**	**M6**
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
10	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ

15	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
20	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
30	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
45	BLQ	BLQ	BLQ	BLQ	BLQ	0.324
60	BLQ	BLQ	BLQ	BLQ	BLQ	0.516
75	BLQ	0.209	0.227	BLQ	BLQ	0.658
90	0.203	0.278	0.289	BLQ	BLQ	0.796
105	0.241	0.280	0.348	BLQ	BLQ	0.910
120	0.261	0.343	0.286	BLQ	BLQ	0.940
150	0.300	0.411	0.344	BLQ	0.200	1.11
180	0.328	0.448	0.349	BLQ	BLQ	1.16
300	0.475	0.395	0.381	BLQ	0.227	1.30
360	0.473	0.417	0.392	BLQ	0.217	1.49
Day 2	BLQ	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

2. Individual raw data showing changes in analyte concentration over time in whole blood (chiral)

Table 7. Changes in **R-mephedrone** concentration over time in M2-M6 in whole blood; Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M2	**M3**	**M4**	**M5**	**M6**
Timepoint (min)	**Concentration** (ng/mL)				
Pre	BLQ	BLQ	BLQ	BLQ	BLQ
5	11.9	46.5	15.3	26.1	89.9
10	18.1	41.0	22.3	30.2	92.7
15	18.1	40.4	26.4	37.6	72.5
20	32.2	35.7	29.4	42.0	51.1
30	33.9	38.3	34.7	54.2	64.2
45	41.0	42.5	38.0	44.1	66.7
60	41.1	38.9	34.3	45.0	63.6
75	40.1	34.5	34.2	36.9	56.3
90	36.3	34.2	30.8	32.7	47.9
105	33.3	28.9	29.4	31.3	41.5
120	32.5	25.1	26.2	28.1	35.4
150	24.9	22.6	24.0	24.0	23.2
180	17.5	20.2	17.7	21.6	15.1
300	9.32	11.2	8.07	9.43	10.8
360	7.18	8.61	6.80	6.85	6.37
Day 2	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	N/C

Table 8. Changes in **S-mephedrone** concentration over time in M2-M6 in whole blood;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M2	M3	M4	M5	M6
Timepoint (min)	**Concentration** (ng/mL)				
Pre	BLQ	BLQ	BLQ	BLQ	BLQ
5	11.7	47.3	14.8	26.3	87.7
10	17.4	41.9	22.3	30.1	90.1
15	17.8	40.6	27.4	37.3	71.9
20	29.1	36.0	28.6	40.4	49.7
30	33.1	35.7	33.0	49.7	63.2
45	36.8	37.9	35.7	39.4	62.6
60	34.7	34.4	31.1	39.2	59.5
75	34.8	29.6	30.8	29.2	52.7
90	31.3	29.5	26.1	24.2	44.8
105	27.6	24.1	25.4	23.7	38.8
120	26.6	21.6	20.8	21.0	32.6
150	20.3	17.8	18.0	17.0	20.7
180	13.7	16.4	12.6	14.3	13.1
300	6.63	7.81	5.10	4.96	8.95
360	4.90	6.35	3.90	3.41	4.73
Day 2	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	N/C

3. Individual raw data showing changes in analyte concentration over time in plasma

Table 9. Changes in **mephedrone** concentration over time in M1-M6 in plasma;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	M2	M3	M4	M5	M6
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	53.3	22.3	65.1	26.8	48.3	131
10	53.5	35.0	69.1	35.4	54.5	140
15	53.4	39.4	56.4	39.5	70.1	127
20	60.4	68.2	53.3	46.0	76.9	103
30	60.5	78.8	66.5	56.4	87.3	152
45	58.7	86.0	65.0	60.3	74.8	162
60	57.8	89.1	62.4	55.1	65.4	169
75	64.4	85.3	50.1	48.0	54.5	171
90	56.9	76.9	47.7	45.2	49.4	130
105	45.6	70.7	46.3	40.1	45.0	111

120	43.9	59.6	43.9	38.3	43.6	94.2
150	40.4	41.9	35.5	32.9	35.5	69.2
180	37.0	29.7	31.2	25.0	29.3	52.1
300	19.5	15.5	17.9	12.1	13.1	32.9
360	N/C	12.8	13.6	9.37	9.64	23.7
Day 2	BLQ	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

Table 10. Changes in **dihydro-mephedrone** concentration over time in M1-M6 in plasma;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	**M2**	**M3**	**M4**	**M5**	**M6**
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	BLQ	0.261
10	BLQ	BLQ	BLQ	BLQ	BLQ	0.356
15	BLQ	BLQ	0.100	BLQ	0.128	0.398
20	0.136	0.103	0.157	BLQ	0.161	0.447
30	0.189	0.179	0.274	0.144	0.253	0.951
45	0.289	0.385	0.699	0.214	0.336	1.69
60	0.408	0.777	0.950	0.253	0.485	2.02
75	0.596	0.862	1.17	0.262	0.550	2.54
90	0.839	1.01	1.55	0.296	0.783	2.75
105	0.869	0.945	1.44	0.308	0.723	2.79
120	0.933	0.943	1.28	0.328	0.779	2.61
150	1.00	1.02	1.35	0.343	0.764	1.97
180	0.990	0.878	1.32	0.349	0.685	1.93
300	0.993	0.703	1.16	0.259	0.523	1.68
360	N/C	0.633	1.02	0.241	0.446	1.32
Day 2	BLQ	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

Table 11. Changes in **nor-mephedrone** concentration over time in M1-M6 in plasma;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	**M2**	**M3**	**M4**	**M5**	**M6**
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	BLQ	0.171
10	BLQ	BLQ	0.189	BLQ	BLQ	0.601
15	0.236	0.193	0.353	0.199	0.182	1.04
20	0.448	0.453	0.855	0.357	0.343	1.37
30	0.887	1.18	1.65	0.909	0.857	3.64
45	1.52	2.82	3.90	1.87	1.38	9.06

60	2.39	4.84	5.52	1.82	2.29	10.9
75	3.56	6.35	6.41	2.26	2.66	15.7
90	4.81	7.14	7.24	2.47	4.14	16.4
105	4.64	6.95	6.68	2.52	6.04	18.3
120	5.11	7.20	6.37	2.73	5.60	16.3
150	5.40	7.26	5.83	2.96	5.86	13.7
180	5.43	6.77	5.31	2.61	2.81	11.3
300	4.81	5.33	4.11	1.64	1.91	8.61
360	N/C	4.60	3.68	1.45	1.78	7.59
Day 2	0.115	BLQ	0.138	BLQ	N/C	N/C
Day 3	BLQ	N/C	0.122	BLQ	BLQ	N/C

Table 12. Changes in **hydroxytolyl-mephedrone** concentration over time in M1-M6 in plasma;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	**M2**	**M3**	**M4**	**M5**	**M6**
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	BLQ	0.186
10	BLQ	BLQ	BLQ	BLQ	BLQ	0.670
15	0.174	0.181	0.101	BLQ	BLQ	0.970
20	0.204	0.318	0.229	0.148	0.150	1.26
30	0.250	0.573	0.356	0.326	0.337	3.24
45	0.369	0.981	0.835	0.348	0.458	4.27
60	0.583	1.24	0.934	0.374	0.662	3.90
75	0.621	1.29	0.868	0.327	0.727	2.91
90	0.851	1.11	0.977	0.338	1.272	2.87
105	0.706	1.05	0.705	0.297	0.653	2.78
120	0.647	0.960	0.535	0.269	0.706	2.17
150	0.511	0.781	0.408	0.312	0.518	1.47
180	0.439	0.710	0.323	0.263	0.155	1.06
300	0.235	0.271	0.159	BLQ	0.100	0.352
360	N/C	0.210	0.120	BLQ	BLQ	0.283
Day 2	BLQ	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

Table 13. Changes in **4-carboxy-mephedrone** concentration over time in M1-M6 in plasma;

Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	**M2**	**M3**	**M4**	**M5**	**M6**
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	0.626	0.243	0.421	17.1
10	4.00	3.36	12.9	5.79	7.08	46.2

15	18.1	16.9	18.0	19.0	17.8	56.5
20	30.6	34.5	41.9	28.5	33.5	78.3
30	44.1	129	102	121	46.9	216
45	49.5	194	125	202	131	542
60	91.8	250	162	198	185	586
75	122	215	150	155	207	795
90	152	187	140	176	272	446
105	126	182	126	186	261	486
120	109	136	106	176	199	505
150	94.8	136	87.2	179	173	198
180	92.5	97.0	66.3	122	48.2	222
300	57.8	42.6	40.3	39.9	38.6	130
360	N/C	31.7	32.9	30.9	31.3	86.2
Day 2	BLQ	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

Table 14. Changes in **dihydro-nor-mephedrone** concentration over time in M1-M6 in plasma; Pre: pre-administration, BLQ: below level of quantification, N/C: not collected

	M1	**M2**	**M3**	**M4**	**M5**	**M6**
Timepoint (min)	**Concentration** (ng/mL)					
Pre	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
5	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
10	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
15	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
20	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
30	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
45	BLQ	BLQ	BLQ	BLQ	BLQ	0.422
60	BLQ	BLQ	0.277	BLQ	BLQ	0.588
75	BLQ	BLQ	0.212	BLQ	BLQ	0.859
90	BLQ	0.205	0.311	BLQ	BLQ	1.00
105	BLQ	0.209	0.318	BLQ	BLQ	1.27
120	BLQ	0.225	0.295	BLQ	BLQ	1.21
150	0.205	0.267	0.333	BLQ	BLQ	1.46
180	0.253	0.295	0.359	BLQ	BLQ	1.26
300	0.307	0.291	0.389	BLQ	BLQ	1.37
360	N/C	0.293	0.408	BLQ	BLQ	1.45
Day 2	BLQ	BLQ	BLQ	BLQ	N/C	N/C
Day 3	BLQ	N/C	BLQ	BLQ	BLQ	N/C

4. Individual plots showing changes in analyte concentrations over time in whole blood

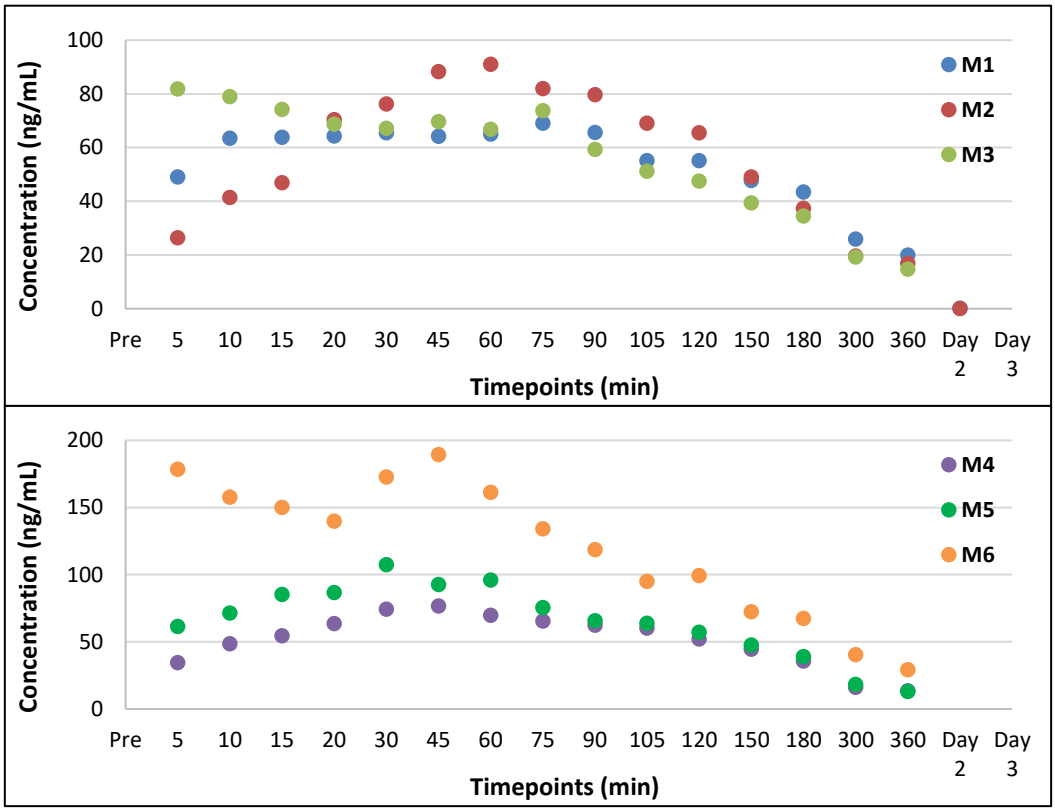


Figure 1. Changes in **mephedrone** concentration over time (above LOQ) in whole blood in M1-M3 (top) and M4-M6 (bottom)

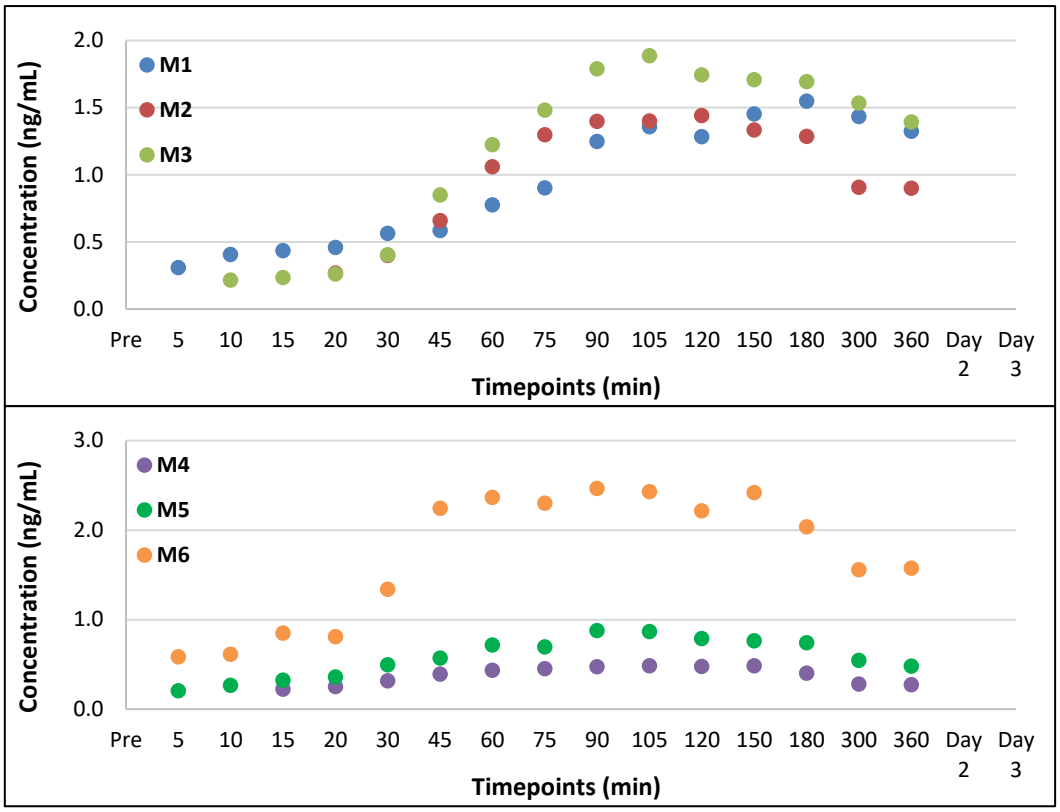


Figure 2. Changes in **dihydro-mephedrone** concentration over time (above LOQ) in whole blood in M1-M3 (top) and M4-M6 (bottom)

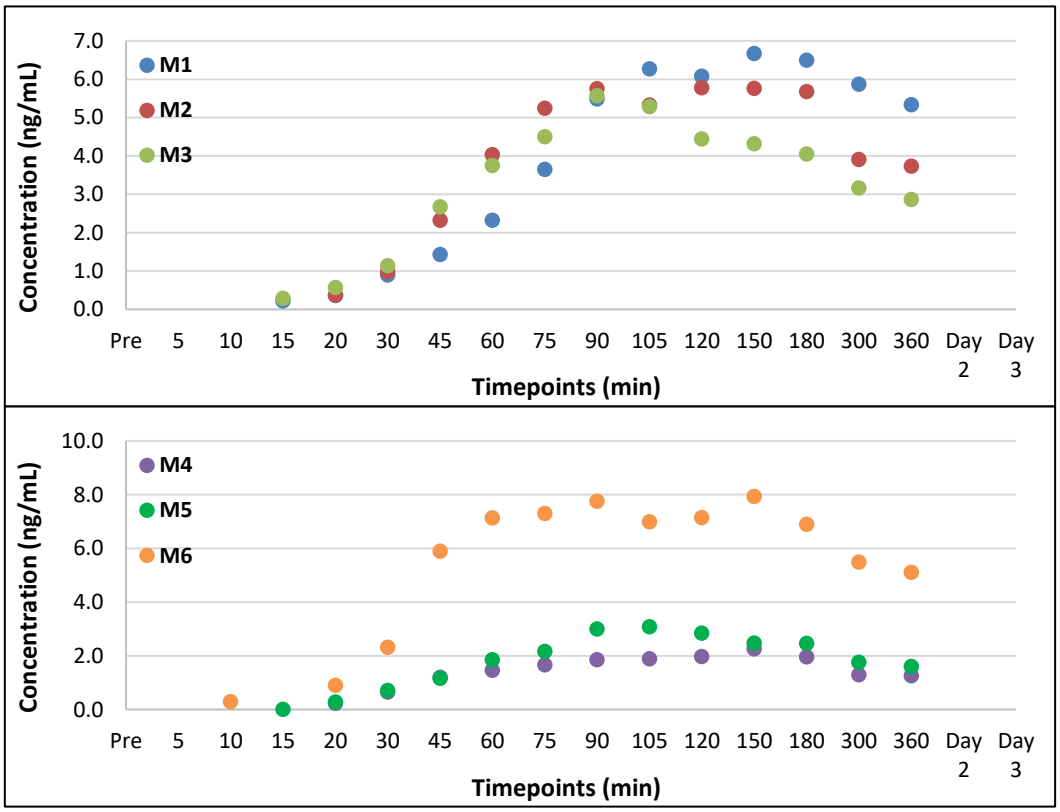


Figure 3. Changes in **nor-mephedrone** concentration over time (above LOQ) in whole blood in M1-M3 (top) and M4-M6 (bottom)

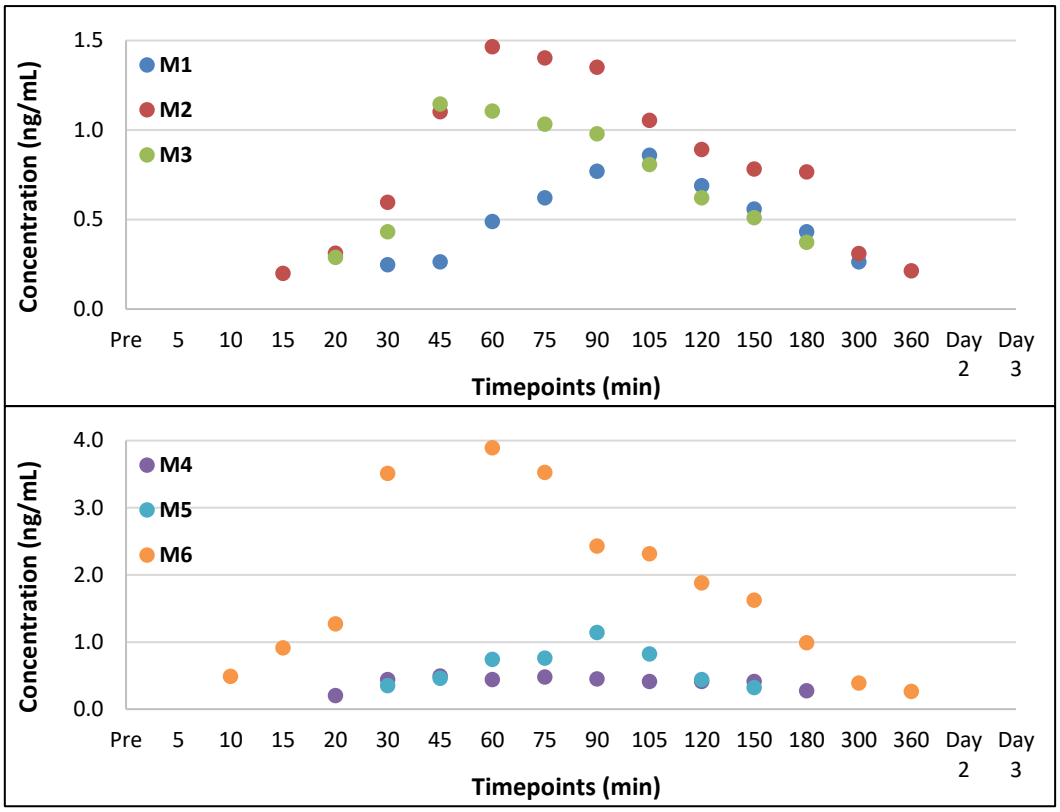


Figure 4. Changes in **hydroxytolyl-mephedrone** concentration over time (above LOQ) in whole blood in M1-M3 (top) and M4-M6 (bottom)

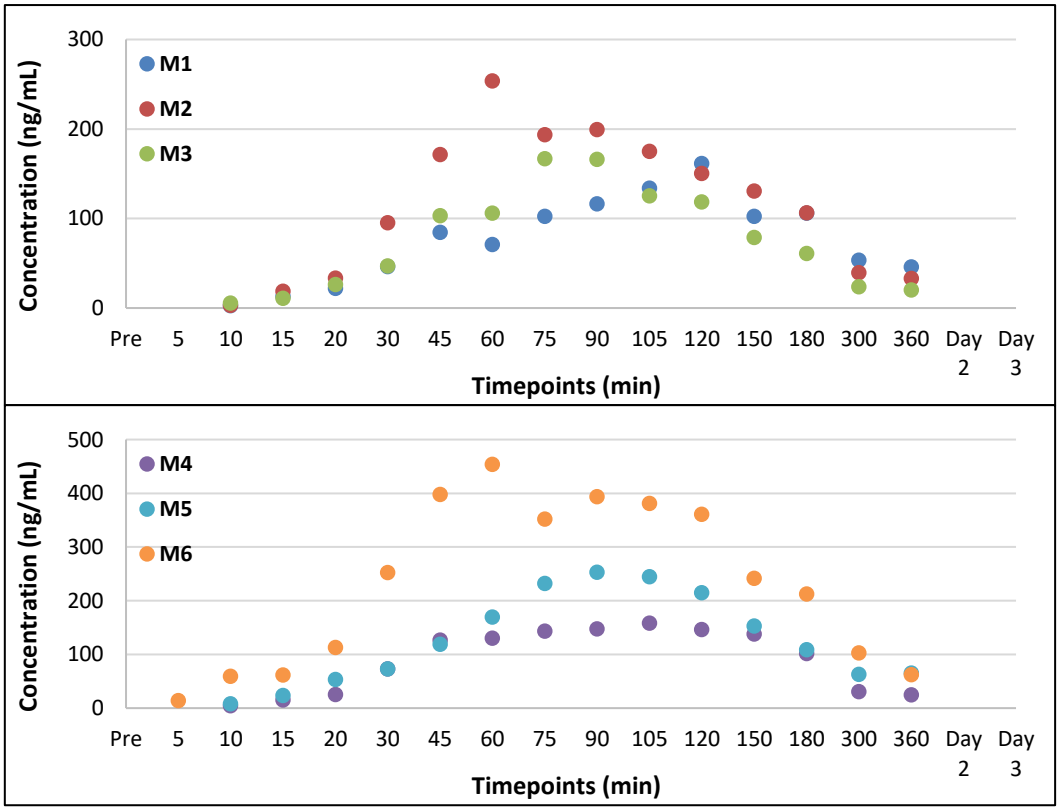


Figure 5. Changes in **4-carboxy-mephedrone** concentration over time (above LOQ) in whole blood in M1-M3 (top) and M4-M6 (bottom)

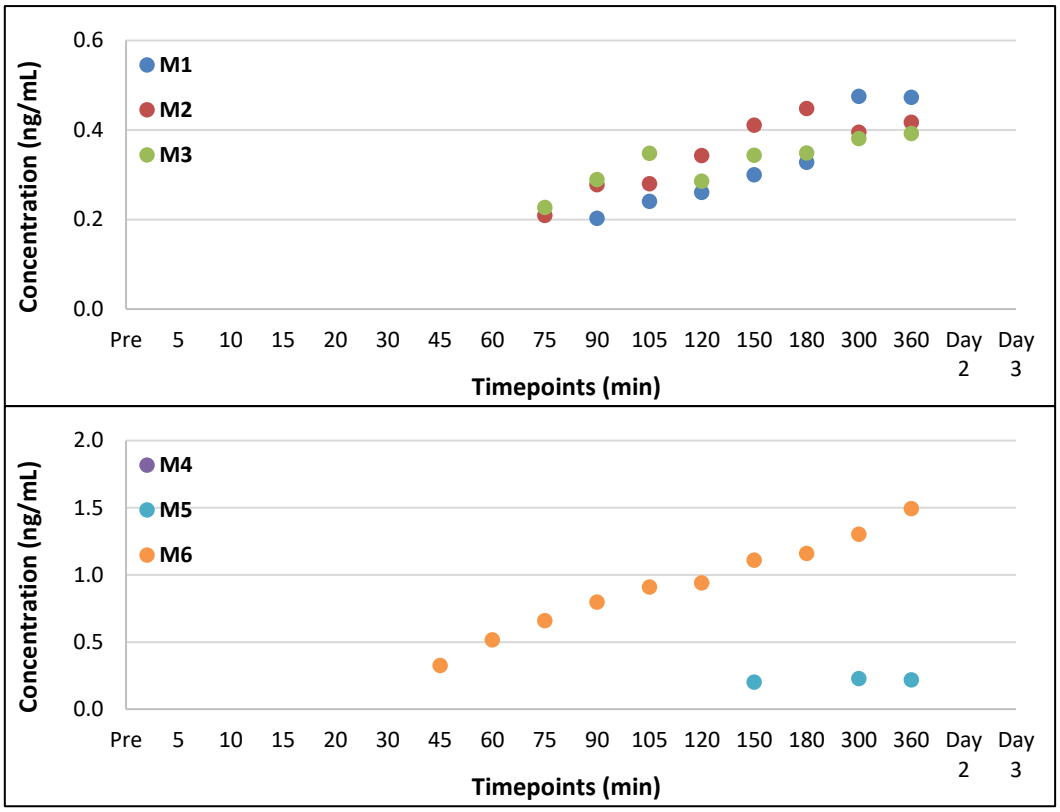


Figure 6. Changes in **dihydro-nor-mephedrone** concentration over time (above LOQ) in whole blood in M1-M3 (top) and M4-M6 (bottom)

5. Individual plots showing changes in analyte concentrations over time in plasma

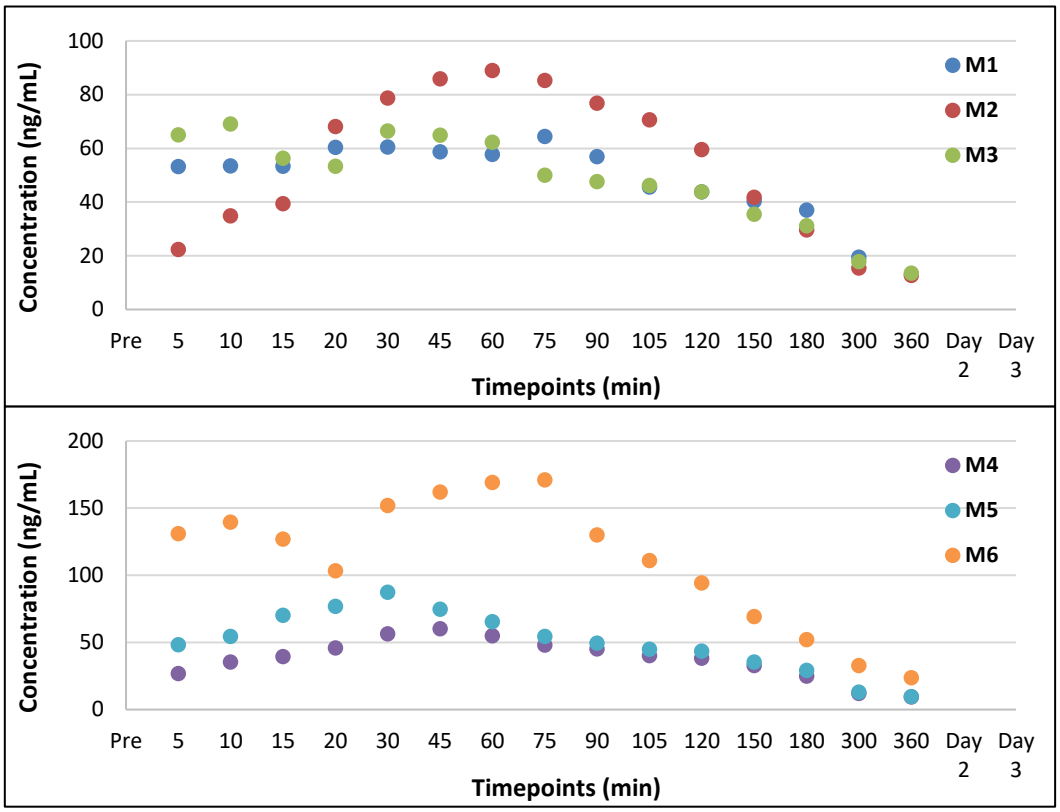


Figure 7. Changes in **mephedrone** concentration over time (above LOQ) in plasma in M1-M3 (top) and M4-M6 (bottom)

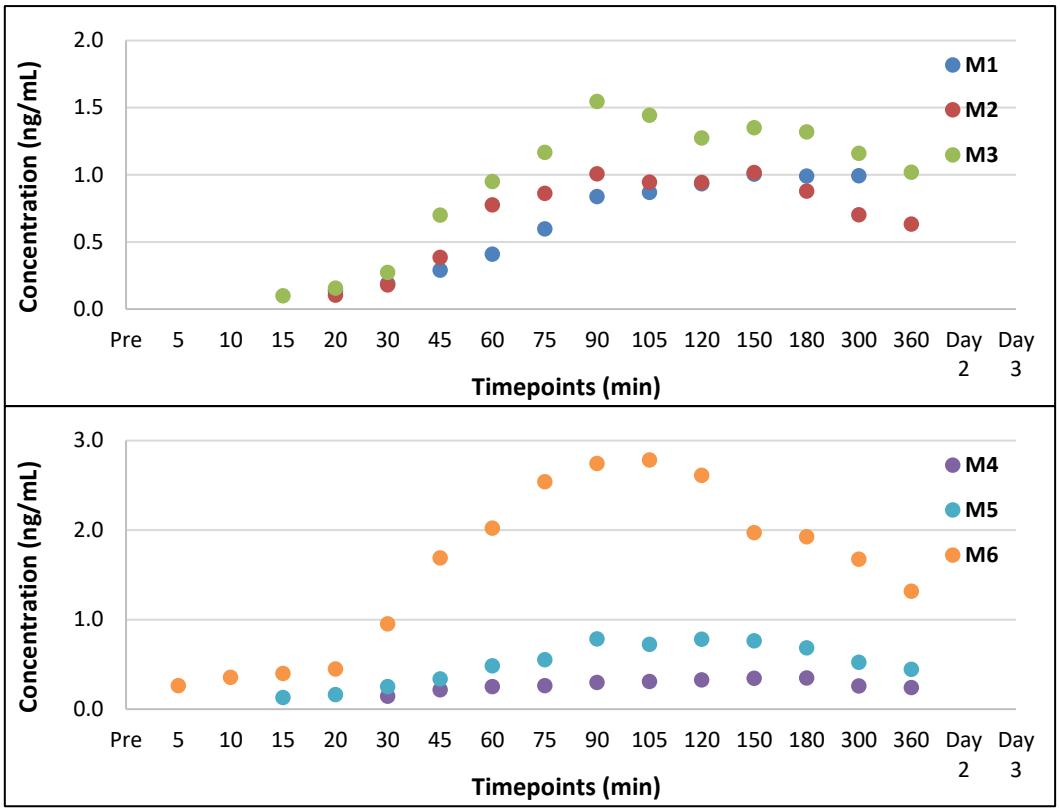


Figure 8. Changes in **dihydro-mephedrone** concentration over time (above LOQ) in plasma in M1-M3 (top) and M4-M6 (bottom)

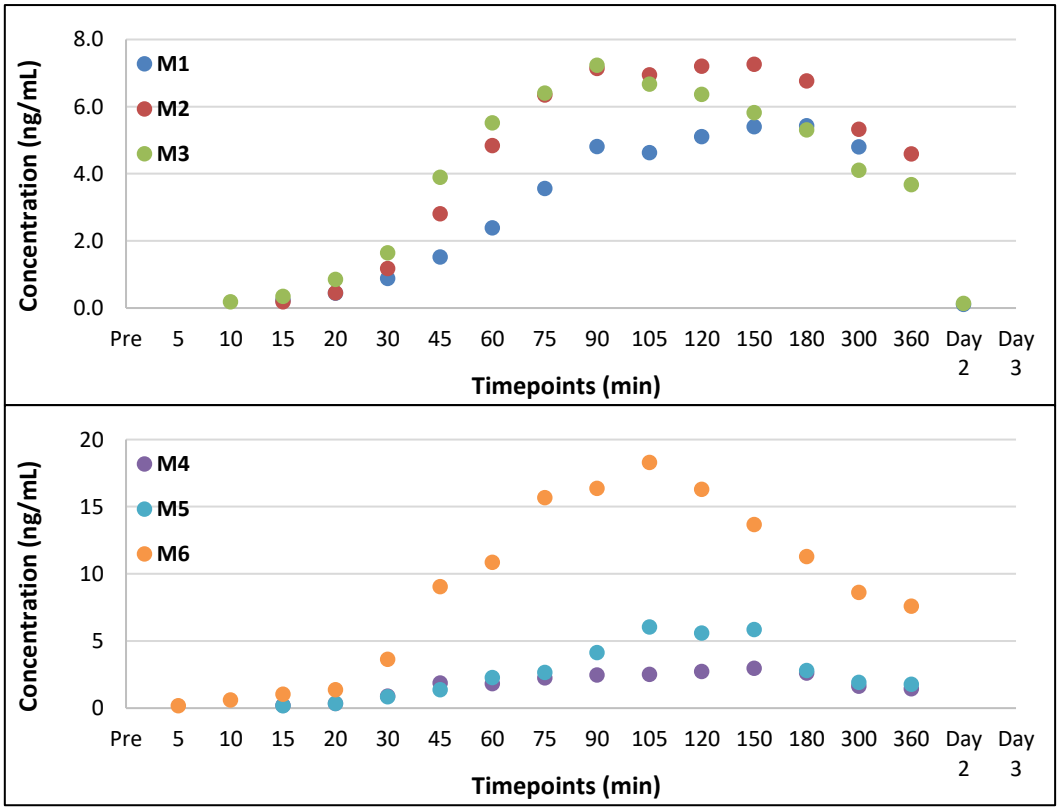


Figure 9. Changes in **nor-mephedrone** concentration over time (above LOQ) in plasma in M1-M3 (top) and M4-M6 (bottom)

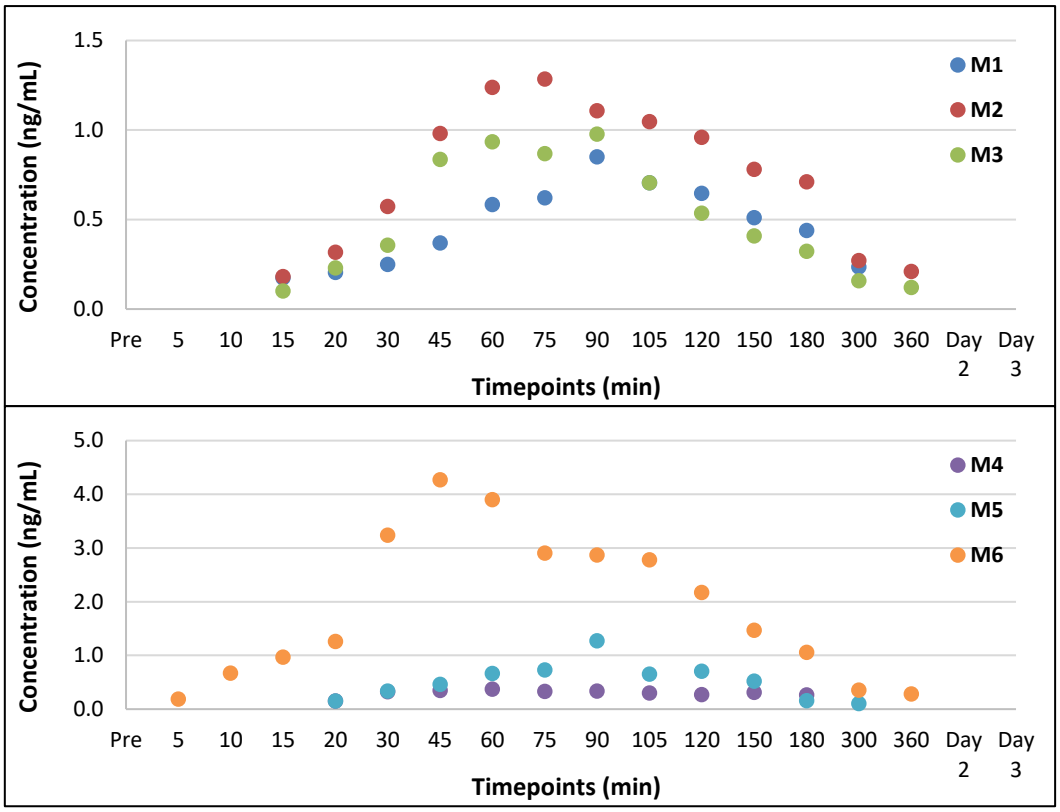


Figure 10. Changes in **hydroxytolyl-mephedrone** concentration over time (above LOQ) in plasma in M1-M3 (top) and M4-M6 (bottom)

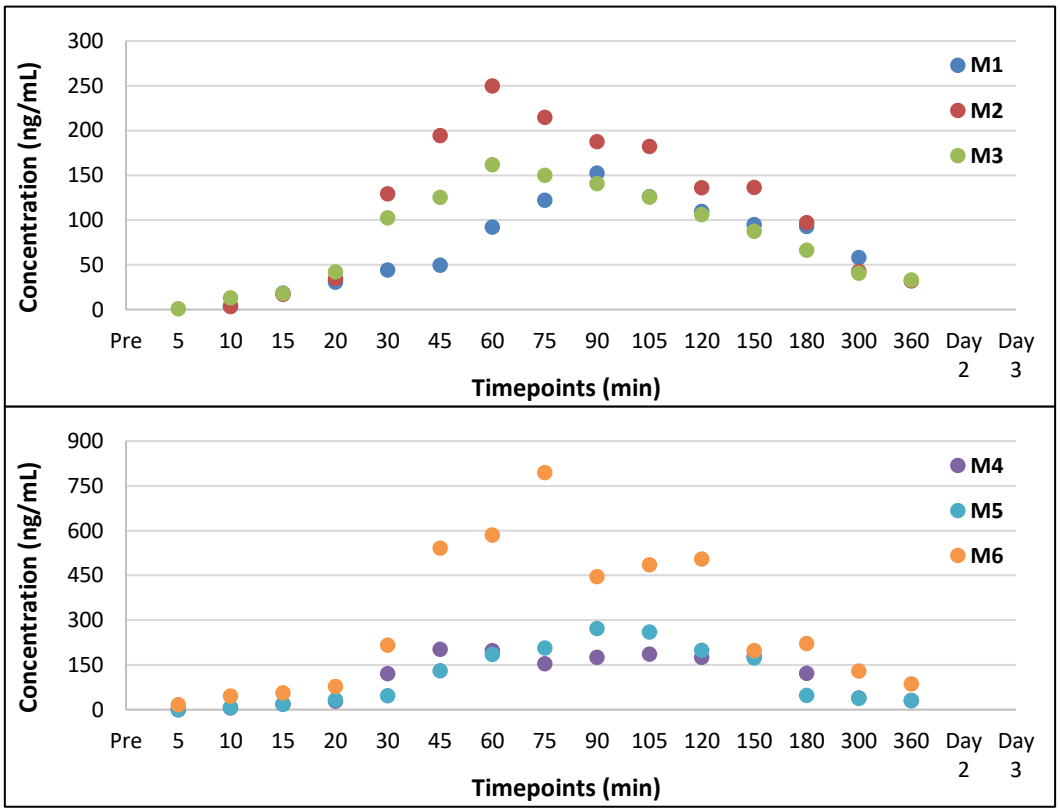


Figure 11. Changes in **4-carboxy-mephedrone** concentration over time (above LOQ) in plasma in M1-M3 (top) and M4-M6 (bottom)

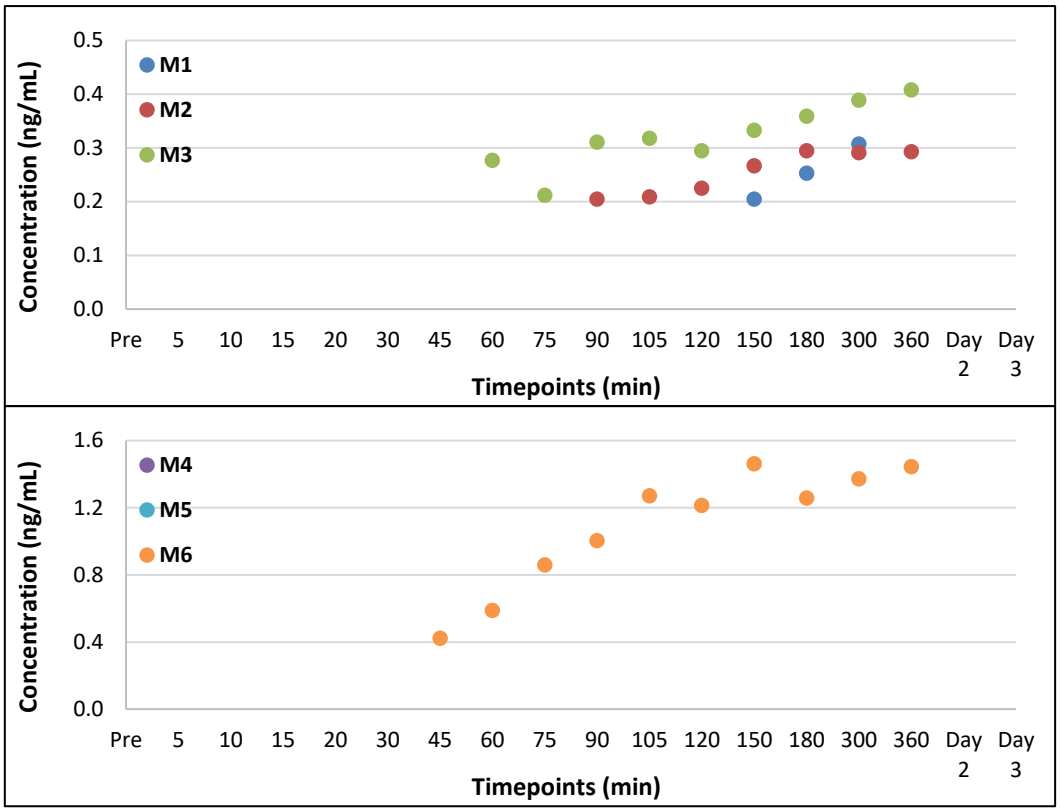


Figure 12. Changes in **dihydro-nor-mephedrone** concentration over time (above LOQ) in plasma in M1 M3 (top) and M4-M6 (bottom)

6. Individual pharmacokinetic data from whole blood

Table 15. Pharmacokinetic data from analysis of **mephedrone** in whole blood from 6 male participants (M1-M6)

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)	CL (mL min^{-1} kg^{-1})	V (L kg^{-1})
M1	69.1	75	0.004	2.74	448	44.2	10.5
M2	91.1	60	0.006	1.94	422	51.9	8.72
M3	73.8	75	0.005	2.19	372	68.9	13.1
M4	76.7	45	0.006	2.00	359	78.2	13.5
M5	108	30	0.006	1.81	475	42.4	6.65
M6	189	45	0.006	2.06	767	39.3	7.01

Table 16. Pharmacokinetic data from analysis of **dihydro-mephedrone** in whole blood from 6 male participants (M1-M6)

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)
M1	1.55	180	0.001	13.9	19.4
M2	1.44	120	0.003	4.47	14.3
M3	1.89	105	0.001	10.9	21.1
M4	0.483	105	0.003	3.35	4.62
M5	0.876	90	0.002	5.20	11.0
M6	2.47	90	0.002	5.34	25.4

Table 17. Pharmacokinetic data from analysis of **nor-mephedrone** in whole blood from 6 male participants (M1-M6)

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)
M1	6.67	150	0.001	11.4	78.3
M2	5.77	150	0.002	5.69	59.4
M3	5.57	90	0.002	4.82	46.7
M4	2.25	150	0.003	4.30	20.3
M5	3.08	105	0.002	4.65	35.9
M6	7.93	150	0.002	5.73	81.4

Table 18. Pharmacokinetic data from analysis of **hydroxytolyl-mephedrone** in whole blood from 6 male participants (M1-M6)

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)
M1	0.860	105	0.006	2.04	2.28
M2	1.47	60	0.006	1.79	5.94
M3	1.15	45	0.009	1.31	2.35
M4	0.493	45	0.005	2.18	1.39
M5	1.14	90	0.021	0.55	1.38
M6	5.38	45	0.009	1.24	11.2

Table 19. Pharmacokinetic data from analysis of **4-carboxy-mephedrone** in whole blood from 6 male participants (M1-M6)

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)
M1	162	120	0.005	2.18	905
M2	254	60	0.007	1.68	905
M3	197	75	0.008	1.47	572
M4	158	105	0.008	1.46	729
M5	253	90	0.007	1.66	1675
M6	454	60	0.006	1.78	1842

Table 20. Pharmacokinetic data from analysis of **dihydro-nor-mephedrone** in whole blood from 6 male participants (M1-M6); N/D: not detected. Note: k_{el} and $t_{1/2}$ were not determined because the elimination phase was not observed from the data

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)
M1	0.475	300	-	-	5.97
M2	0.448	180	-	-	5.64
M3	0.392	360	-	-	5.23
M4	N/D	N/D	N/D	N/D	N/D
M5	0.227	300	-	-	4.06
M6	1.49	360	-	-	19.3

7. Individual pharmacokinetic data from plasma

Table 21. Pharmacokinetic data from analysis of **mephedrone** in plasma from 6 male participants (M1-M6)

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)	CL (mL min^{-1} kg^{-1})	V (L kg^{-1})
M1	64.4	75	0.005	2.35	392	50.5	10.2
M2	89.1	60	0.007	1.66	360	60.8	8.74
M3	66.5	30	0.005	2.36	333	77.0	15.7
M4	60.3	45	0.006	1.92	257	109.1	18.2
M5	87.3	30	0.007	1.77	354	56.9	8.73
M6	171	75	0.006	1.81	674	44.7	7.00

Table 22. Pharmacokinetic data from analysis of **dihydro-mephedrone** in plasma from 6 male participants (M1-M6); k_{el} and $t_{1/2}$ were not determined in M1 because not enough data points were observed in the elimination phase

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)
M1	1.00	150	-	-	13.3
M2	1.02	150	0.002	420	10.1
M3	1.55	90	0.001	506	15.8
M4	0.35	180	0.002	328	3.77
M5	0.78	90	0.002	314	10.03
M6	2.79	105	0.003	251	22.7

Table 23. Pharmacokinetic data from analysis of **nor-mephedrone** in plasma from 6 male participants (M1-M6)

Analyte	C_{max} (ng/mL)	T_{max} (min)	k_{el} (min^{-1})	$t_{1/2}$ (h)	AUC (ng mL^{-1} h)
M1	5.43	180	0.003	3.68	68.2
M2	7.26	150	0.002	5.41	73.8
M3	7.24	90	0.002	5.98	63.8
M4	2.96	150	0.003	3.32	25.0

M5	6.04	105	0.005	2.26	43.4
M6	18.3	105	0.003	3.46	131

Table 24. Pharmacokinetic data from analysis of **hydroxytolyl-mephedrone** in plasma from 6 male participants (M1-M6)

Analyte	**C_{max}** (ng/mL)	**T_{max}** (min)	**k_{el}** (min^{-1})	**$t_{1/2}$** (h)	**AUC** (ng mL^{-1} h)
M1	0.851	90	0.006	1.96	4.39
M2	1.29	75	0.006	1.78	5.63
M3	0.977	90	0.007	1.76	3.38
M4	0.374	60	0.003	3.99	1.09
M5	1.27	90	0.012	1.00	1.90
M6	4.27	45	0.009	1.27	11.3

Table 25. Pharmacokinetic data from analysis of **4-carboxy-mephedrone** in plasma from 6 male participants (M1-M6)

Analyte	**C_{max}** (ng/mL)	**T_{max}** (min)	**k_{el}** (min^{-1})	**$t_{1/2}$** (h)	**AUC** (ng mL^{-1} h)
M1	152	90	0.004	3.10	961
M2	250	60	0.007	1.68	895
M3	162	60	0.005	2.11	744
M4	202	45	0.007	1.77	922
M5	272	90	0.009	1.36	1044
M6	795	75	0.007	1.64	2358

Table 26. Pharmacokinetic data from analysis of **dihydro-nor-mephedrone** in plasma from 6 male participants (M1-M6); N/D: not detected. Note: k_{el} and $t_{1/2}$ were not determined in M1, M2, M3 and M6 because the elimination phase was not observed from the data

Analyte	**C_{max}** (ng/mL)	**T_{max}** (min)	**k_{el}** (min^{-1})	**$t_{1/2}$** (h)	**AUC** (ng mL^{-1} h)
M1	0.307	300	-	-	3.64
M2	0.295	180	-	-	3.91
M3	0.389	300	-	-	5.46

M4	N/D	N/D	N/D	N/D	N/D
M5	N/D	N/D	N/D	N/D	N/D
M6	1.45	360	-	-	19.6

8. Individual VAS data

Table 27. Changes in **VAS "drug effect"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	10	3.0	7.5	1.5	7.0	10	7.3
30	5.0	3.0	2.5	1.5	3.0	10	3.0
45	2.5	5.0	1.0	3.5	1.0	10	3.0
60	2.0	3.0	1.0	1.0	0	10	1.5
75	1.0	0.5	1.0	0.5	0	10	0.8
90	0.5	0.5	0	0.5	0	8.0	0.5
105	0.2	0.5	0	0	0	6.0	0.1
120	0.1	0.5	0	0	0	5.0	0.1
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 28. Changes in **VAS "stimulated"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	9.0	3.0	7.0	4.0	8.0	4.0	5.5
30	6.0	4.5	2.0	3.5	4.0	4.5	4.3
45	3.0	5.0	1.0	2.5	0.5	4.5	2.8
60	2.0	2.0	0.5	1.0	0	0	0.8
75	1.0	1.0	0.5	0.5	0	0	0.5
90	0.5	0.5	0	0.5	0	0	0.3
105	0.5	0.1	0	0.5	0	0	0.1
120	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0

300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 29. Changes in **VAS "high"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	7.0	2.0	6.0	1.0	8.0	10	6.5
30	4.0	4.0	1.5	1.0	5.0	10	4.0
45	4.0	4.5	1.0	3.0	0.5	10	3.5
60	3.0	3.0	0.5	0.5	0.5	10	1.8
75	1.0	3.0	0.5	0.5	0	10	0.8
90	0.5	3.0	0	0.5	0	8.0	0.5
105	0.5	1.0	0	0	0	7.0	0.3
120	0	0.5	0	0	0	6.0	0
150	0	0.5	0	0	0	4.0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 30. Changes in **VAS "liking"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	6.0	2.0	6.0	5.5	9.5	10	6.0
30	6.0	1.5	1.5	4.0	6.0	10	5.0
45	6.0	1.0	1.0	4.0	4.0	10	4.0
60	5.5	5.0	0	3.0	4.0	10	4.5
75	5.0	4.0	0	0	4.0	10	4.0
90	5.0	2.0	0	0	4.0	10	3.0
105	3.0	0.5	0	0	4.0	8.0	1.8
120	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 31. Changes in **VAS "dizzy"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	0.5	1.0	5.0	4.0	2.0	4.0	3.0
30	0	2.0	3.5	3.0	1.0	0	1.5
45	0	2.0	2.0	1.0	0	0	0.5
60	0	0.5	0	1.0	0	0	0
75	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0
105	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 32. Changes in **VAS "good drug effect"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	7.5	4.0	1.0	1.0	10	10	5.8
30	4.0	4.5	1.0	1.0	6.0	10	4.3
45	4.0	5.0	0	3.0	0	10	3.5
60	2.0	2.0	0	0.5	0	10	1.3
75	1.0	2.0	0	0	0	10	0.5
90	0.5	2.0	0	0	0	9.0	0.3
105	0.2	1.0	0	0	0	6.0	0.1
120	0	0	0	0	0	6.0	0
150	0	0	0	0	0	6.0	0
180	0	0	0	0	0	4.0	0
300	0	0	0	0	0	0	0
360	0	0	`	0	0	0	0

Table 33. Changes in **VAS "confused"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	3.0	0.5	2.0	0.5	0.5	6.0	1.3
30	0	1.0	1.0	0.5	0.5	4.0	0.8
45	0	1.0	1.0	0.5	0.5	4.5	0.8
60	0	0	0	0	0	4.0	0
75	0	0	0	0	0	4.0	0
90	0	0	0	0	0	3.0	0
105	0	0	0	0	0	1.5	0
120	0	0	0	0	0	1.5	0
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 34. Changes in **VAS "fearful"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	0	1.0	0	0.5	0.5	1.5	0.5
30	0	1.0	0	0.5	0.5	0	0.3
45	0	1.0	0	0	0.5	0	0
60	0	0	0	0	0	0	0
75	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0
105	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 35. Changes in **VAS "changes in lights"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0
30	0	1.0	1.0	0	0	0	0
45	0	2.5	0.5	0	0	0	0
60	0	0	3.5	0	0	0	0
75	0	0	1.5	0	0	0	0
90	0	0	0	0	0	0	0
105	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 36. Changes in **VAS "changes in distance"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	1.0	0	1.0	1.5	0	0	0.5
30	0	1.0	0	1.0	0	0	0
45	0	2.0	0	2.5	0	0	0
60	0	0.5	0	0.5	0	0	0
75	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0
105	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 37. Changes in **VAS "changes in hearing"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	0	0	0	2.5	0	4.0	0
30	0	0	0	1.5	0	4.5	0
45	0	2.5	0	0.5	0	0	0
60	0	1.0	0	0.5	0	0	0
75	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0
105	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 38. Changes in **VAS "changes in body sensations"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	3.5	4.5	8	2.5	9.0	4.0	4.3
30	1.0	5.5	1.0	3.0	5.0	4.0	3.5
45	1.0	6.0	1.0	4.5	0	4.5	2.8
60	0	2.5	0.5	0.5	0	4.5	0.5
75	0	2.5	0.5	0	0	4.0	0.3
90	0	0.5	0	0	0	1.0	0
105	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

Table 39. Changes in **VAS "changes in surrounding"**, expressed as cm on the VAS scale, after internasal mephedrone administration

Timepoint	M1	M2	M3	M4	M5	M6	Median
Pre	0	0	0	0	0	0	0
15	2.5	1.0	0.5	3.0	0	0	0.8
30	1.0	1.0	0	1.0	0.5	0	0.8
45	0.5	3.0	0	2.5	0	0	0.3
60	0	1.0	0	0.5	0	0	0
75	0	0	0	0	0	0	0
90	0	0	0	0	0	0	0
105	0	0	0	0	0	0	0
120	0	0	0	0	0	0	0
150	0	0	0	0	0	0	0
180	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0
360	0	0	0	0	0	0	0

9. Individual blood pressure and heart rate results

Table 40. Changes in systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) after mephedrone administration in M1 and M2

	M1			M2		
Timepoint (min)	**SBP** (mmHg)	**DBP** (mmHg)	**HR** (bpm)	**SBP** (mmHg)	**DBP** (mmHg)	**HR** (bpm)
Pre	136	85	88	134	79	94
5	160	96	98	148	84	128
10	175	88	125	140	100	140
15	145	89	133	141	90	140
20	144	93	96	141	92	139
30	140	85	92	142	92	157
45	145	82	88	140	89	133
60	140	83	93	140	91	132
75	142	87	86	118	70	125
90	135	77	82	130	80	126
105	132	73	78	128	81	115
120	126	81	74	128	88	116
140	132	76	76	120	71	116
150	140	73	72	120	68	115

170	117	71	69	109	67	119
180	133	67	73	124	69	110
200	129	82	82	122	68	100
220	127	70	77	117	53	95
240	125	65	78	119	67	100
260	127	70	72	111	61	98
280	126	73	73	110	67	98
300	124	76	73	119	65	99
320	121	63	70	120	76	102
340	129	76	71	123	69	92
360	125	67	77	123	76	101

Table 41. Changes in systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) after mephedrone administration in M3 and M4

M3				**M4**		
Timepoint (min)	**SBP** (mmHg)	**DBP** (mmHg)	**HR** (bpm)	**SBP** (mmHg)	**DBP** (mmHg)	**HR** (bpm)
Pre	139	74	68	103	65	75
5	155	87	73	126	81	84
10	144	81	84	125	78	71
15	141	84	82	124	73	97
20	139	88	77	125	75	90
30	136	71	71	121	74	68
45	133	89	68	116	69	70
60	132	78	68	124	74	69
75	122	70	66	113	69	68
90	119	72	63	112	68	69
105	117	73	65	107	69	61
120	120	76	53	107	71	68
140	122	63	54	110	71	68
150	115	76	62	105	74	69
170	109	64	55	109	64	65
180	120	74	62	106	78	63
200	124	73	56	114	76	56
220	118	76	58	118	73	63
240	111	61	61	110	74	63
260	110	55	58	110	69	68
280	102	54	61	108	67	69
300	106	49	61	108	64	66

320	106	60	66	109	65	67
340	103	54	63	110	65	68
360	110	67	63	111	68	68

Table 42. Changes in systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) after mephedrone administration in M5 and M6

	M5			**M6**		
Timepoint (min)	**SBP** (mmHg)	**DBP** (mmHg)	**HR** (bpm)	**SBP** (mmHg)	**DBP** (mmHg)	**HR** (bpm)
Pre	121	75	81	124	82	99
5	159	91	105	142	100	152
10	120	87	105	152	108	151
15	153	96	106	167	108	149
20	153	83	110	151	93	140
30	146	86	97	150	105	140
45	123	79	89	148	93	128
60	136	79	79	147	90	127
75	122	67	65	132	90	109
90	124	74	68	131	91	110
105	121	69	64	131	91	111
120	129	73	60	128	88	114
140	108	64	70	127	84	112
150	112	65	64	129	86	113
170	120	79	61	124	85	109
180	125	73	64	121	81	104
200	127	69	65	120	80	105
220	133	61	74	119	77	104
240	119	64	70	117	79	102
260	126	65	73	121	78	103
280	125	69	76	119	74	94
300	123	76	85	128	78	96
320	118	67	86	122	76	99
340	116	73	82	123	80	98
360	117	71	81	126	80	100

10. Individual correlation between whole blood and plasma concentrations

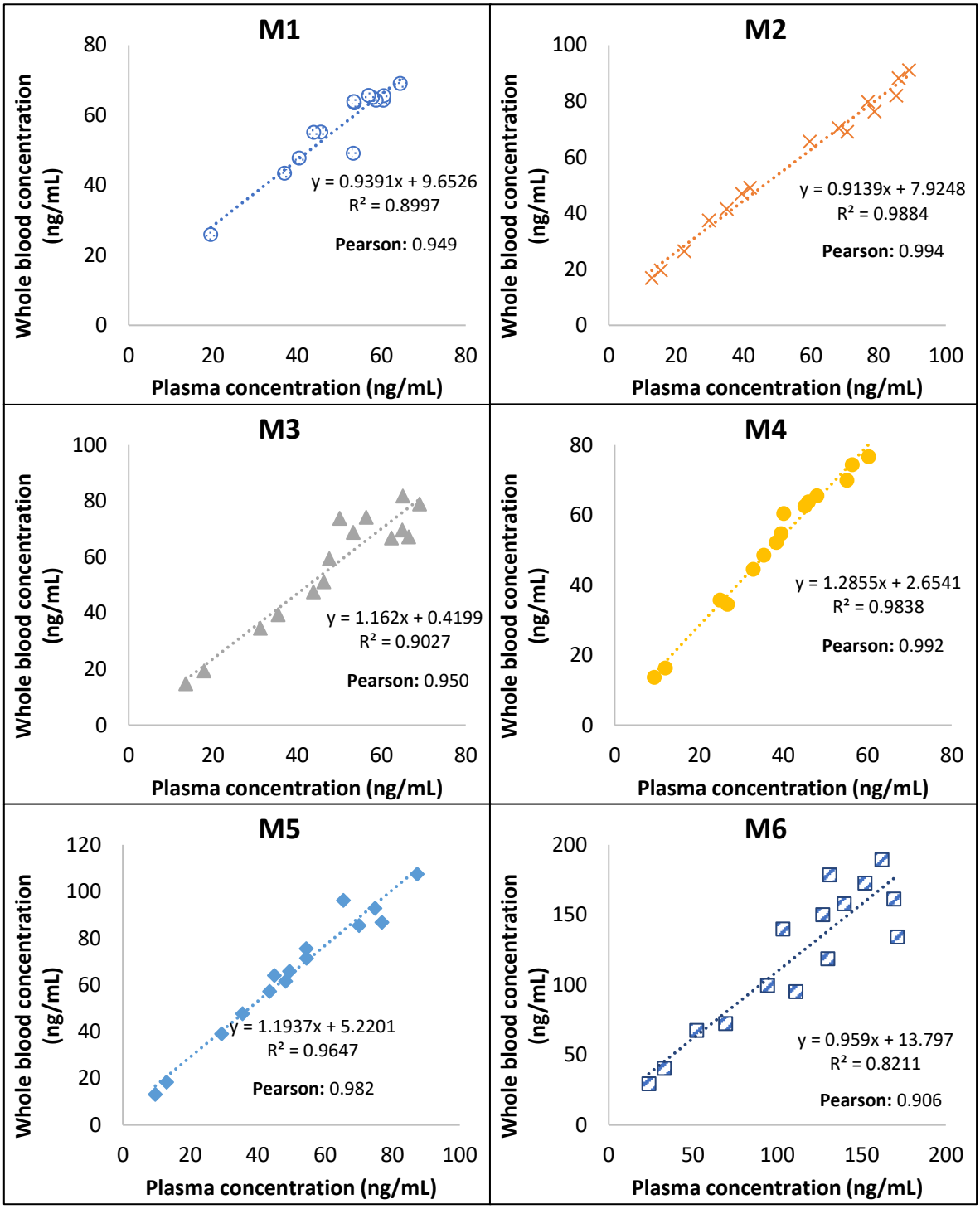


Figure 13. Correlation between whole blood and plasma concentrations for **mephedrone** in M1-M6

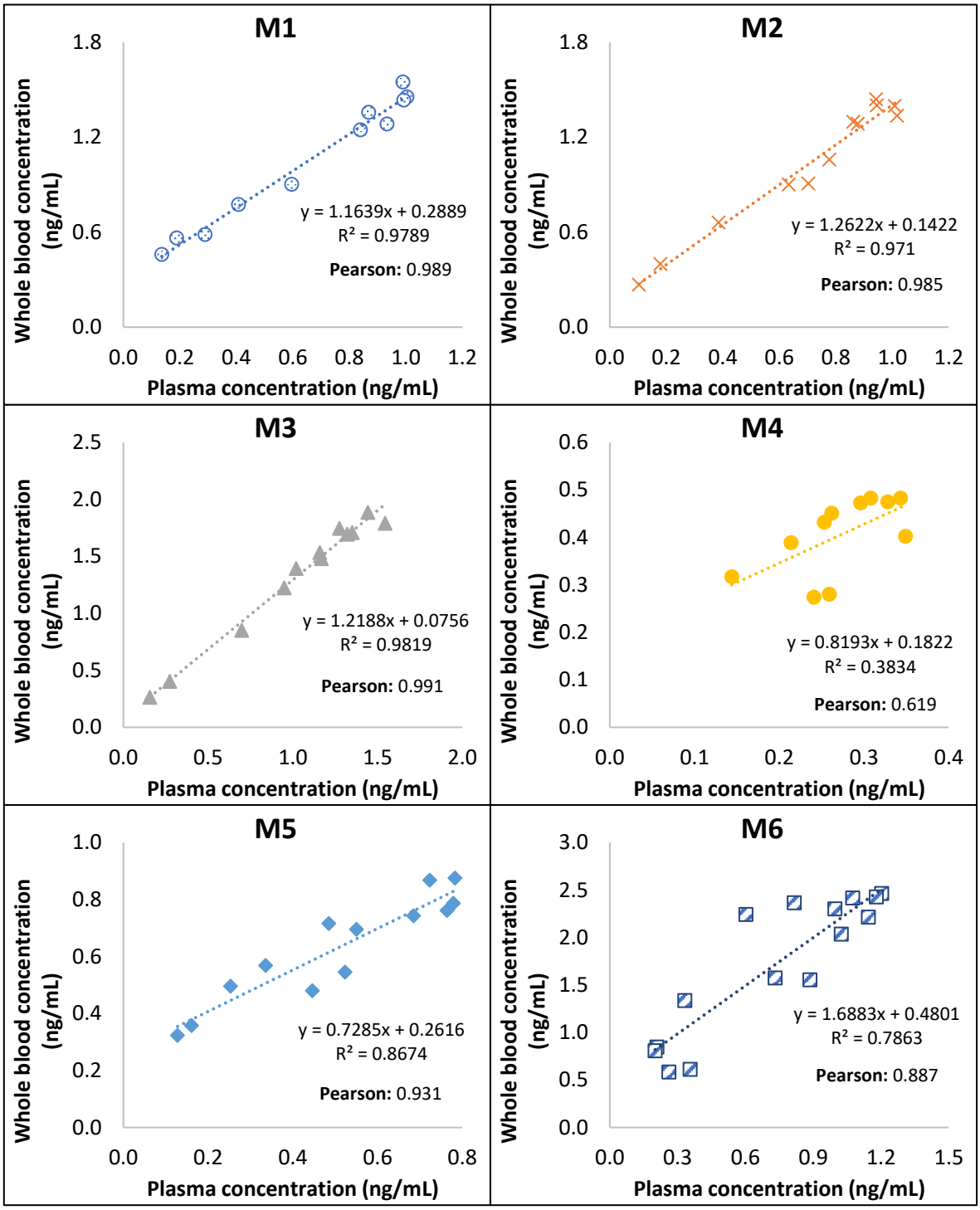


Figure 14. Correlation between whole blood and plasma concentrations for **dihydro-mephedrone** in M1-M6

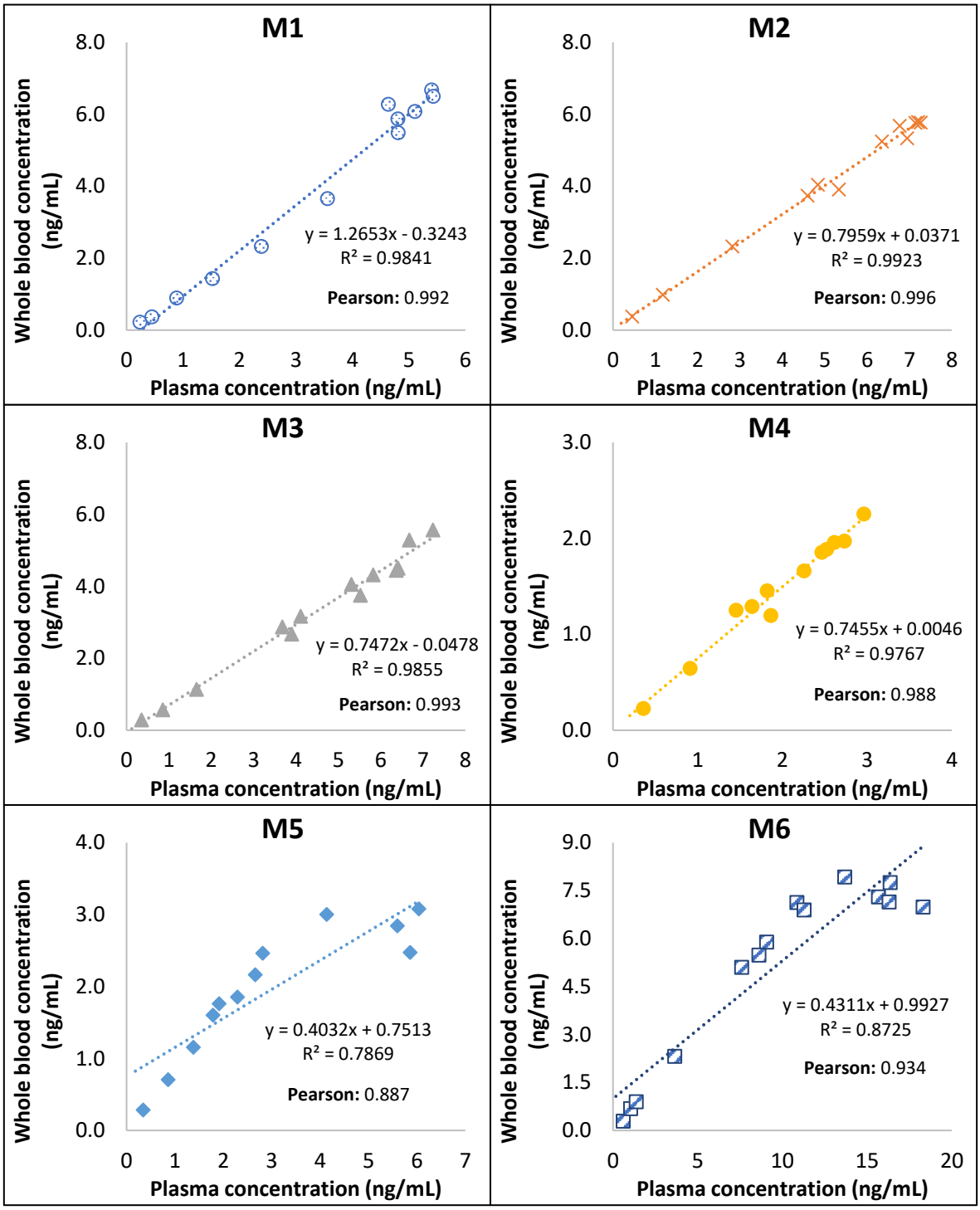


Figure 15. Correlation between whole blood and plasma concentrations for **nor-mephedrone** in M1-M6

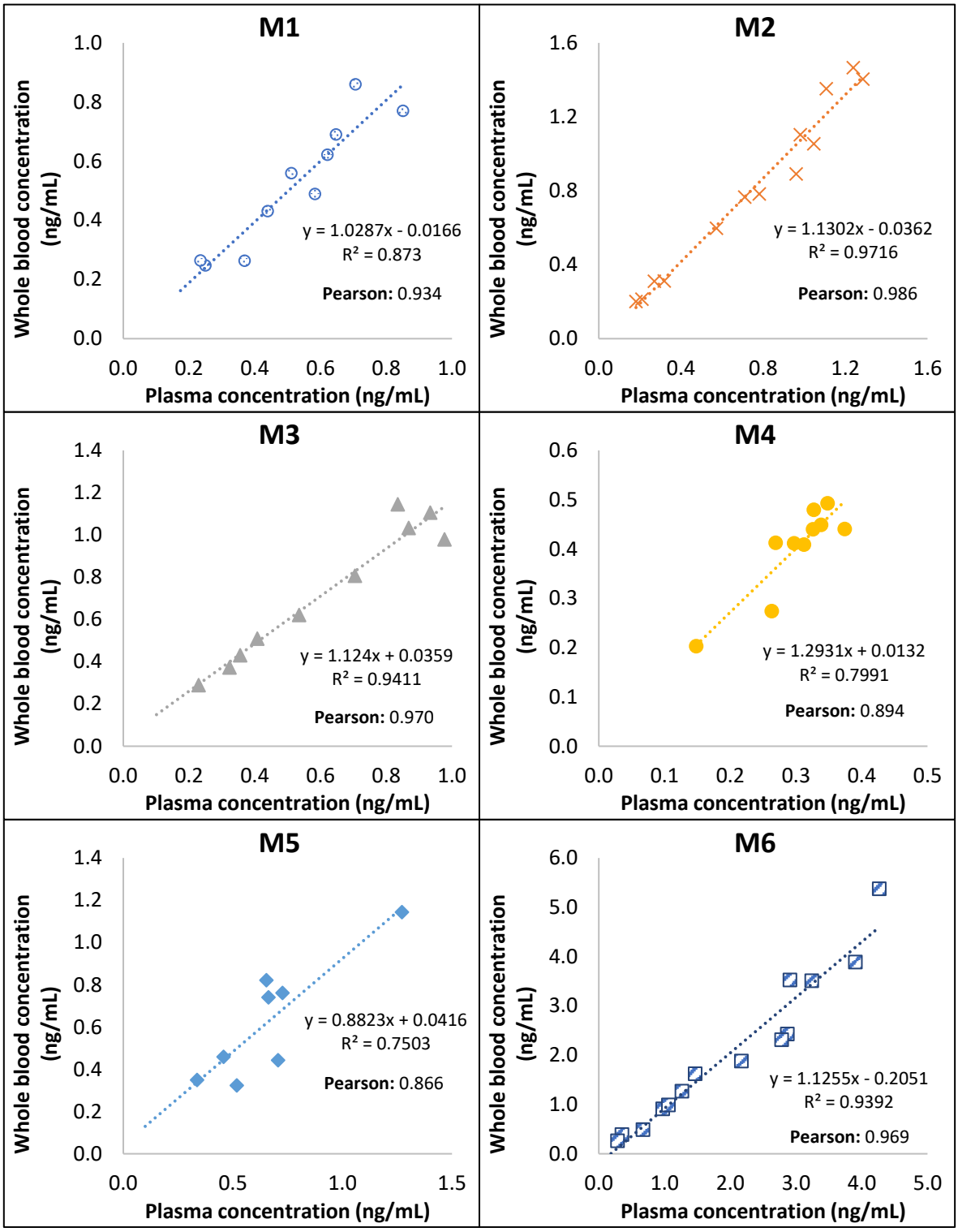


Figure 16. Correlation between whole blood and plasma concentrations for **hydroxytolyl-mephedrone** in M1-M6

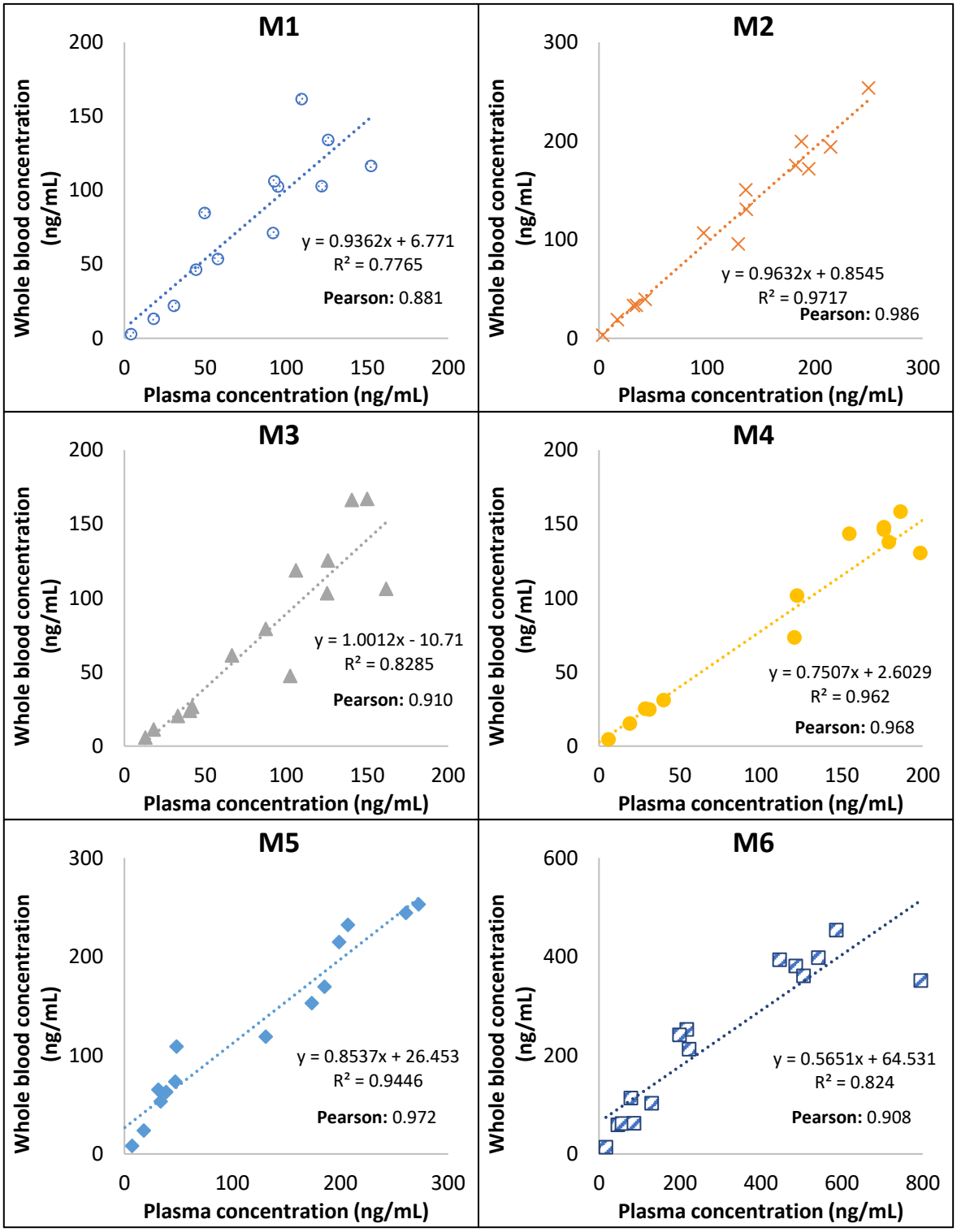


Figure 17. Correlation between whole blood and plasma concentrations for **4-carboxy-mephedrone** in M1-M6

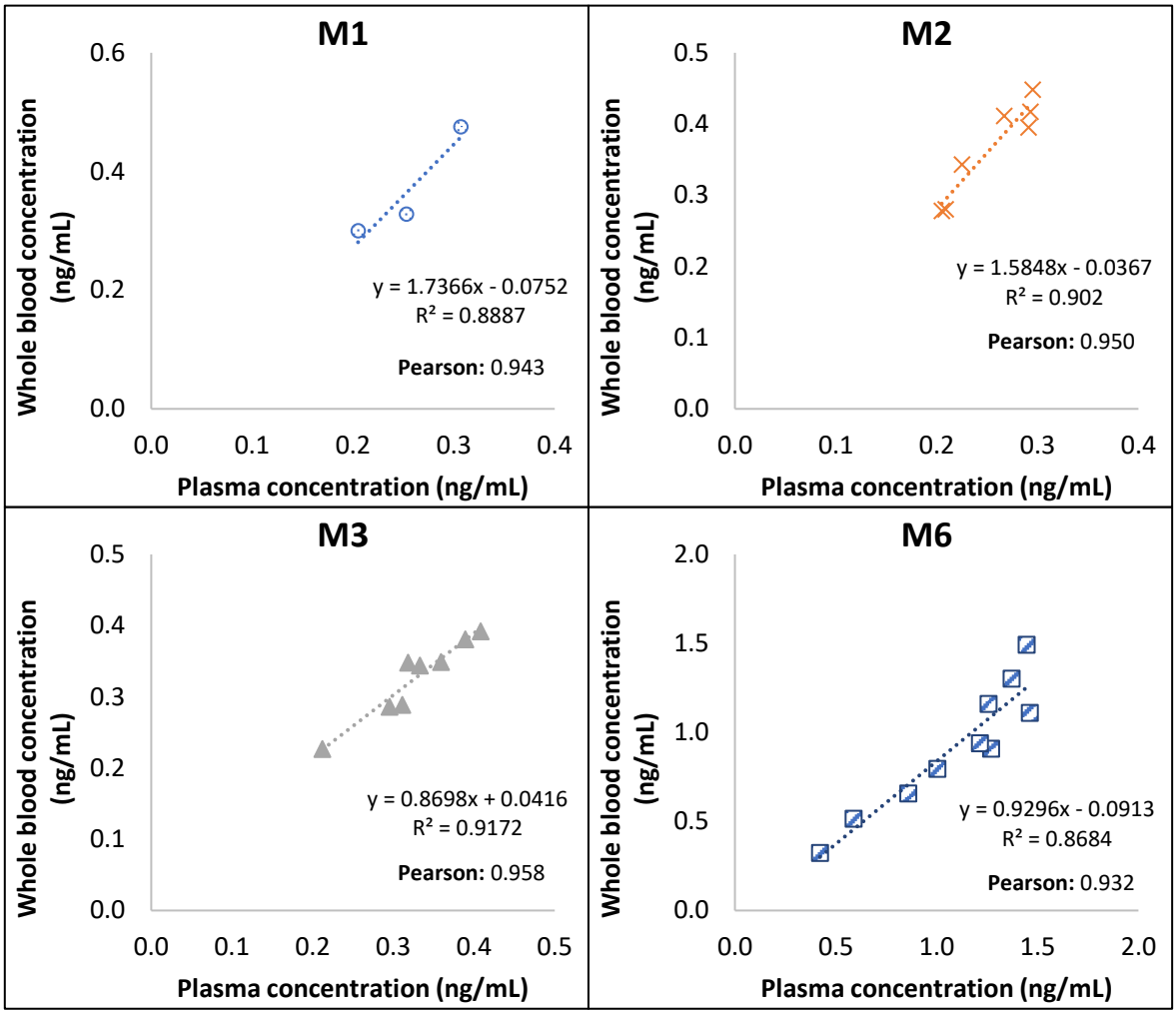


Figure 18. Correlation between whole blood and plasma concentrations for **dihydro-nor-mephedrone** in M1-M6; the analyte was not detected in M4 and M5 (plasma)

11. Individual correlation between DBS and whole blood

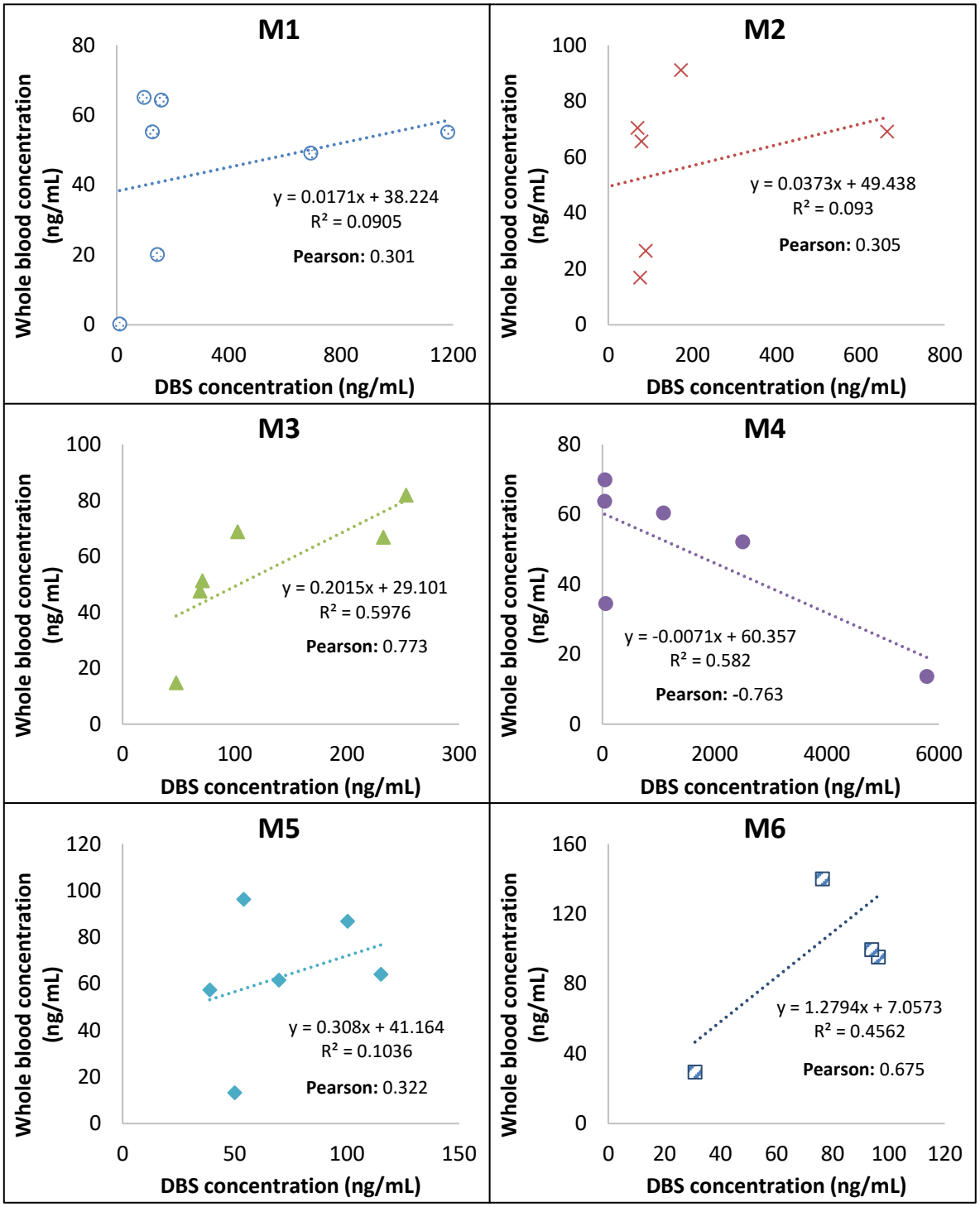


Figure 19. Correlation between whole blood and DBS concentrations for **mephedrone** in M1-M6

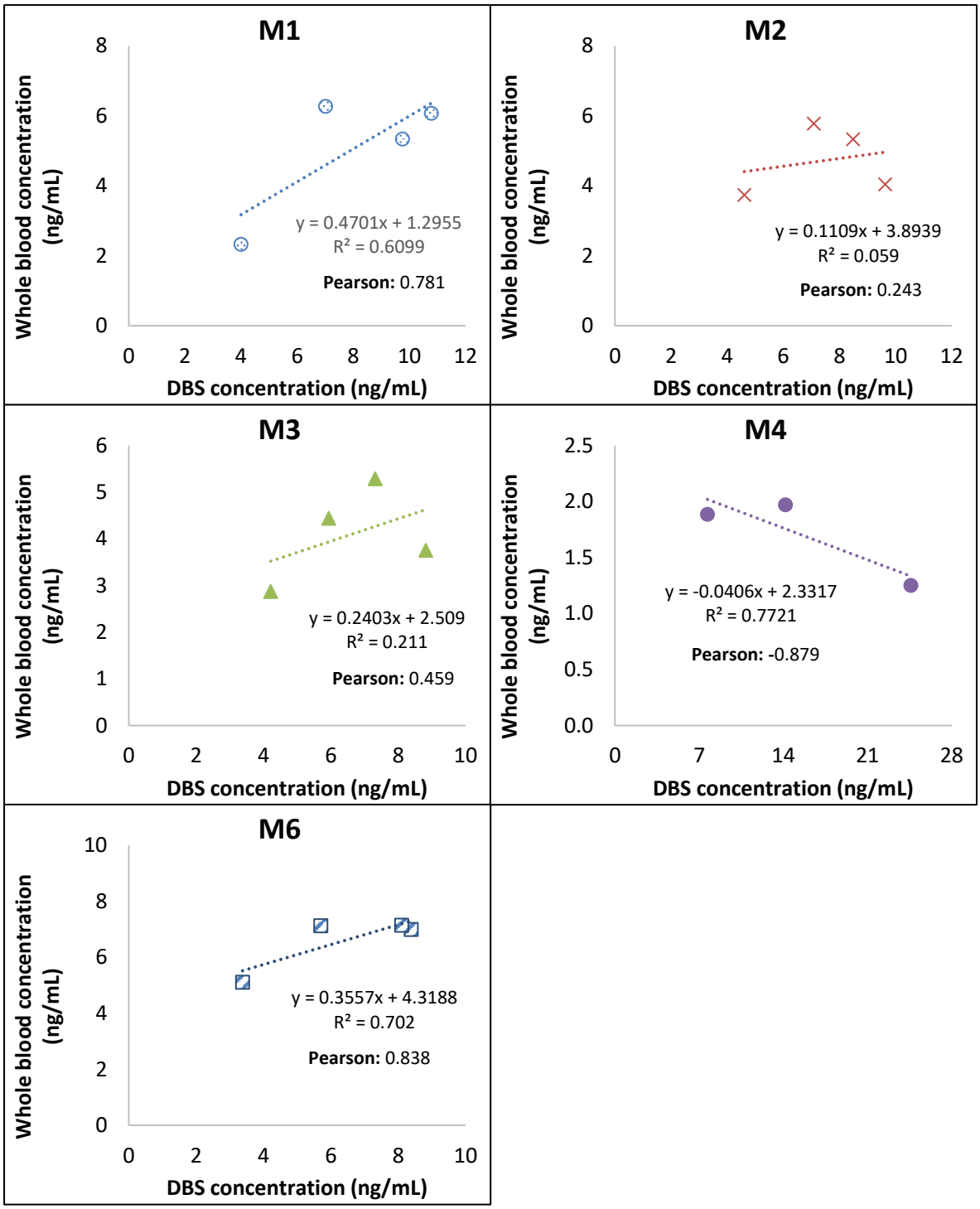


Figure 20. Correlation between whole blood and DBS concentrations for **nor-mephedrone** in M1-M6; the analyte was not detected in DBS in M5

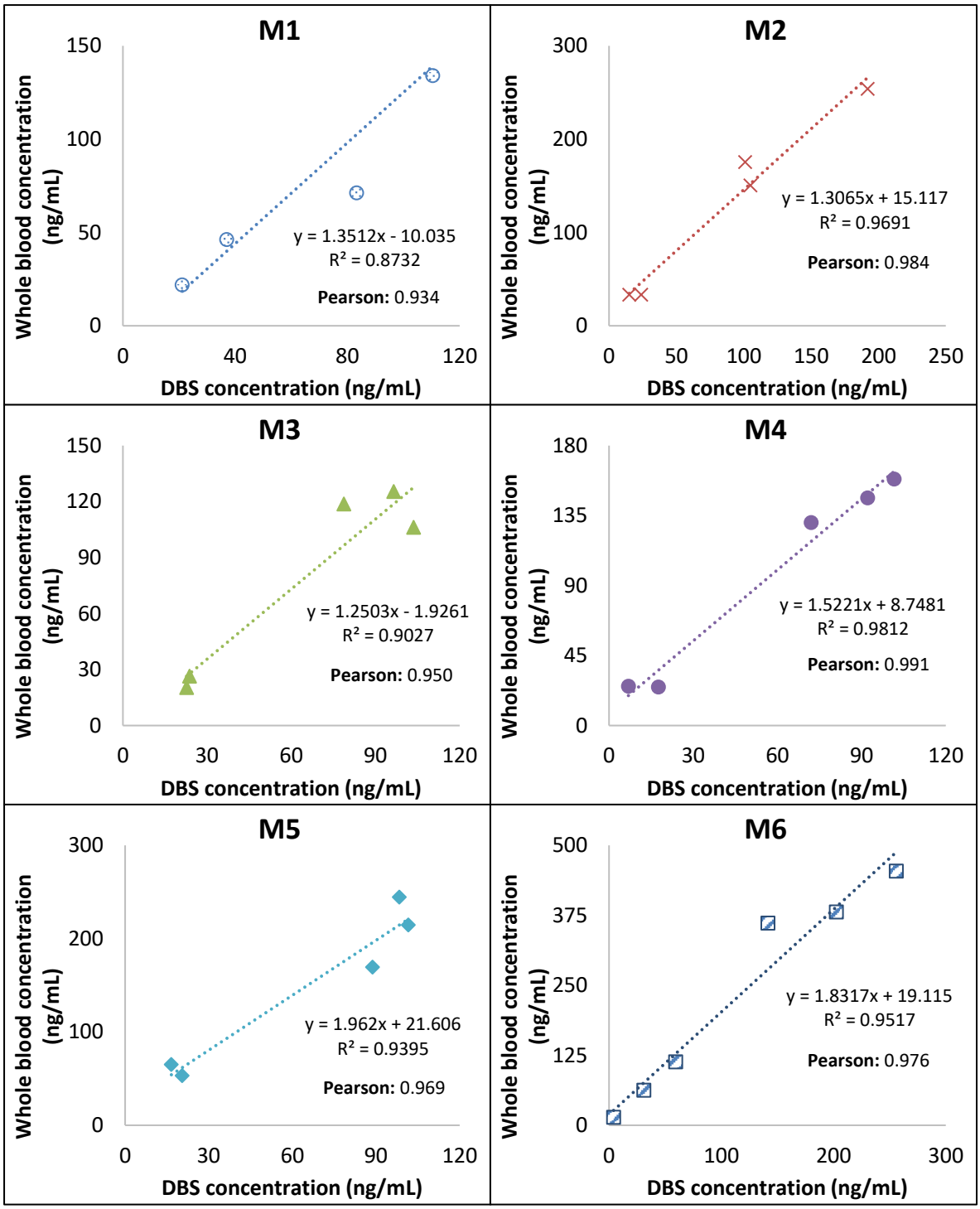


Figure 21. Correlation between whole blood and DBS concentrations for **4-carboxy-mephedrone** in M1-M6

12. Individual correlation between DBS and whole blood

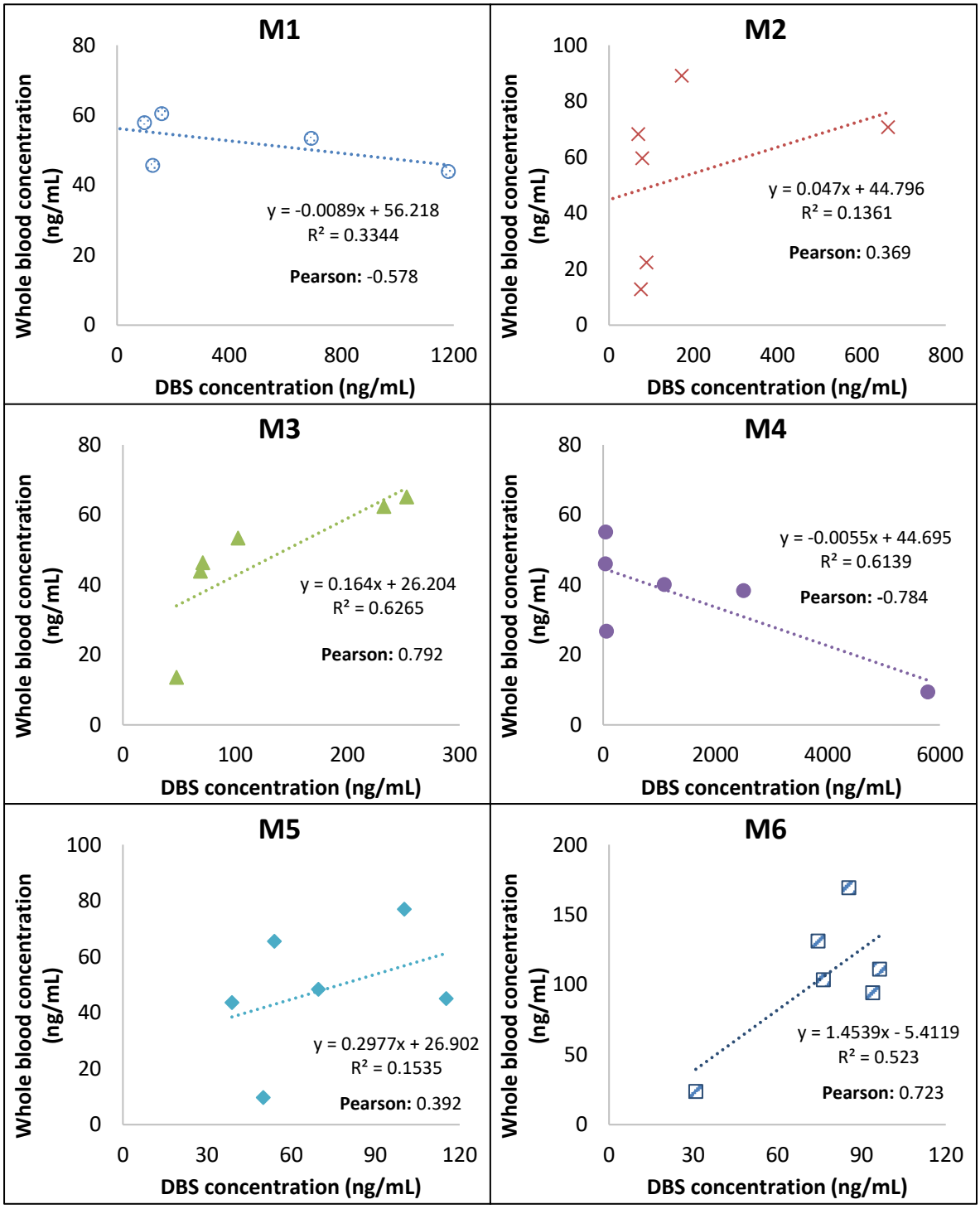


Figure 22. Correlation between plasma and DBS concentrations for **mephedrone** in M1-M6

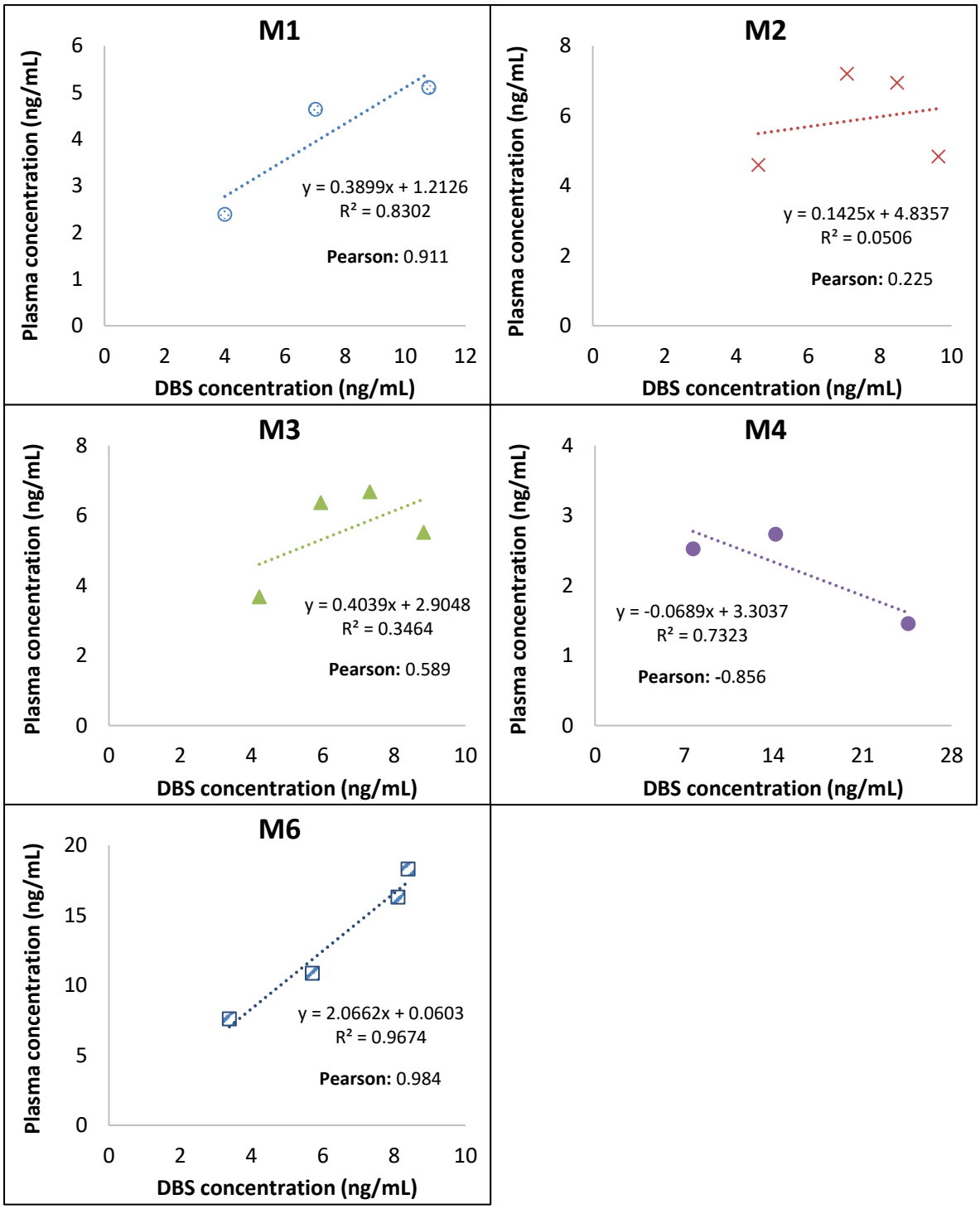


Figure 23. Correlation between plasma and DBS concentrations for **nor-mephedrone** in M1-M6; the analyte was not detected in DBS in M5

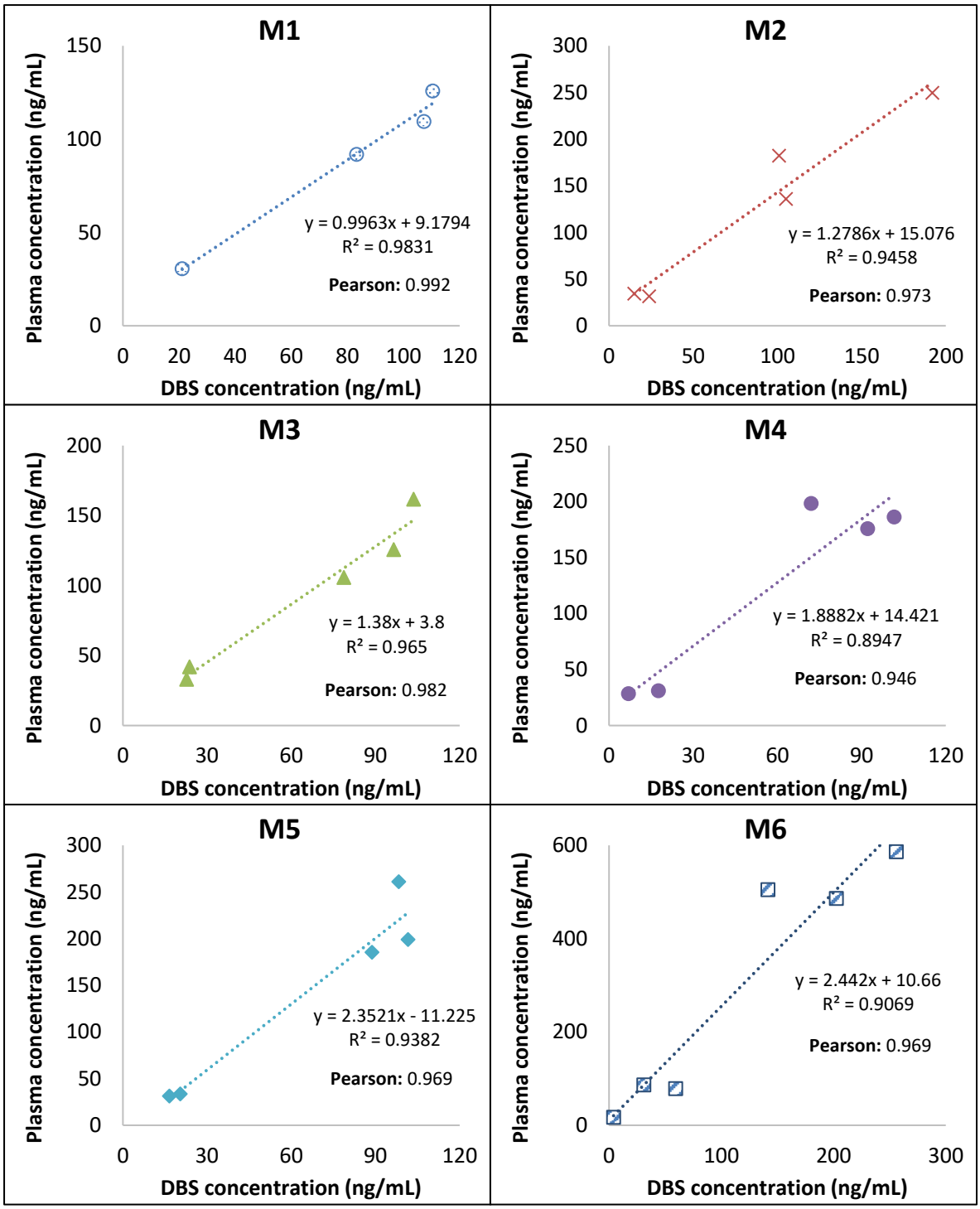


Figure 24. Correlation between plasma and DBS concentrations for **4-carboxy-mephedrone** in M1-M6

13. Individual correlation between whole blood and oral fluid concentration

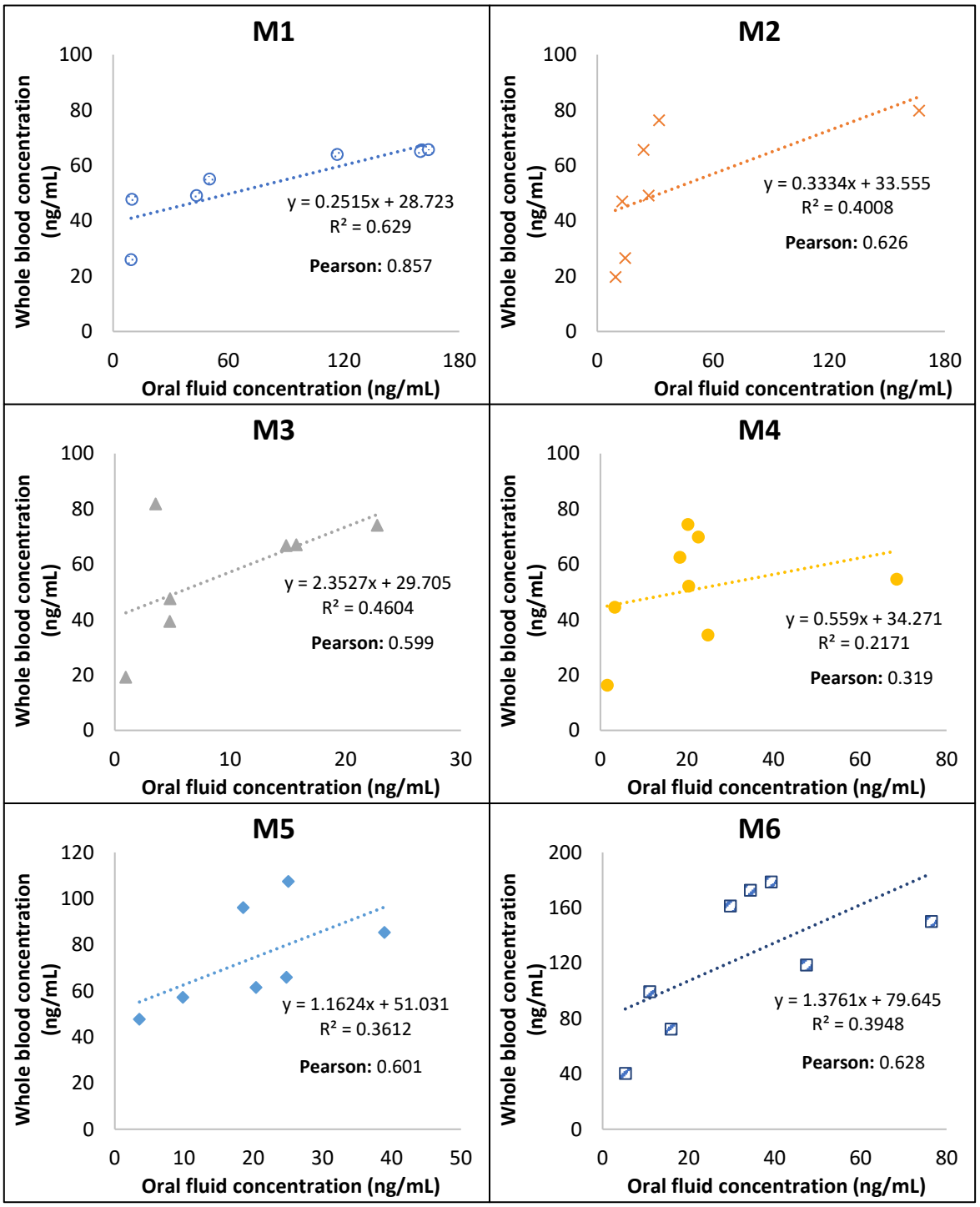


Figure 25. Correlation between oral fluid and whole blood concentrations for **mephedrone** in M1-M6

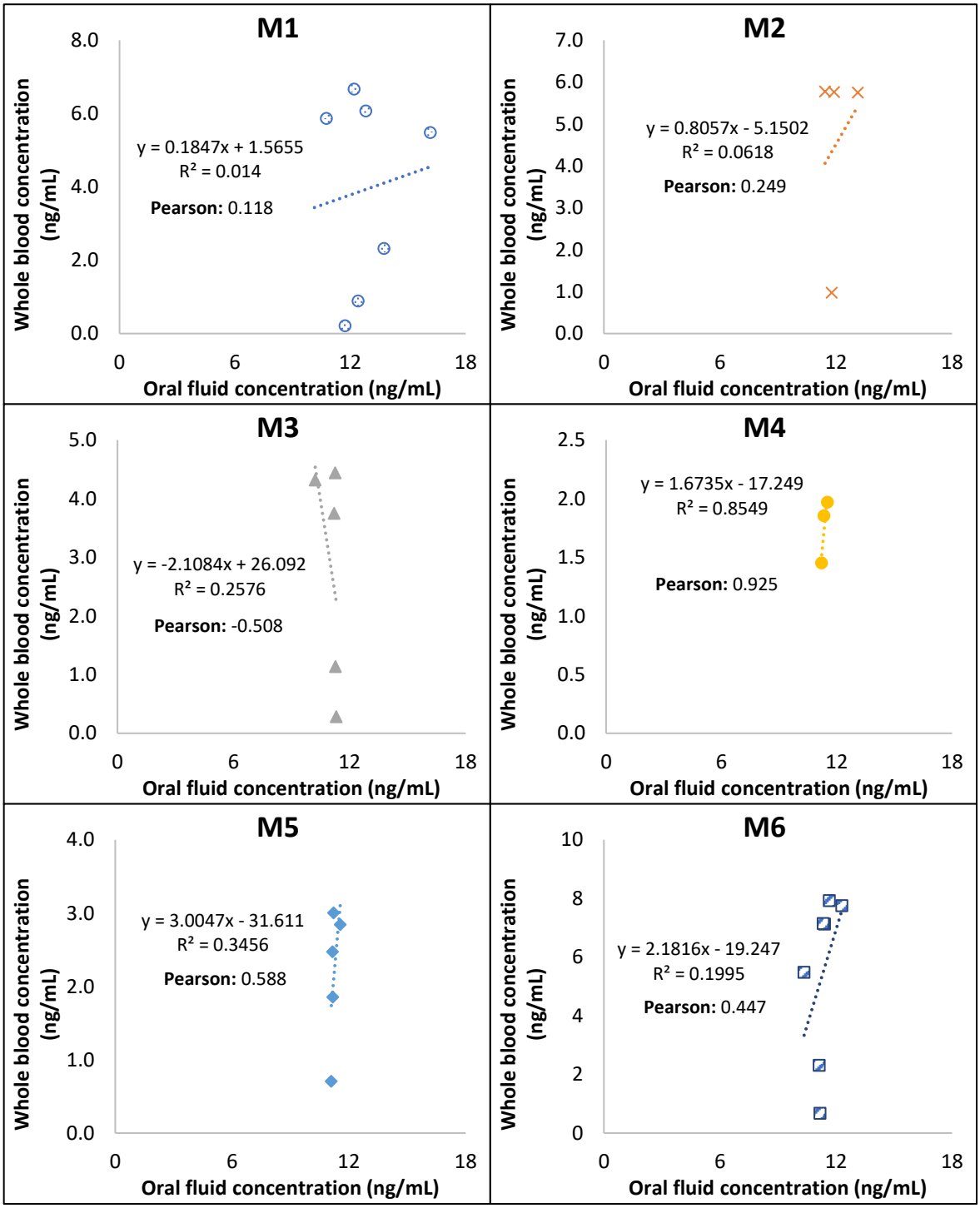


Figure 26. Correlation between oral fluid and whole blood concentrations for **nor-mephedrone** in M1-M6

14. Individual correlation between plasma and oral fluid

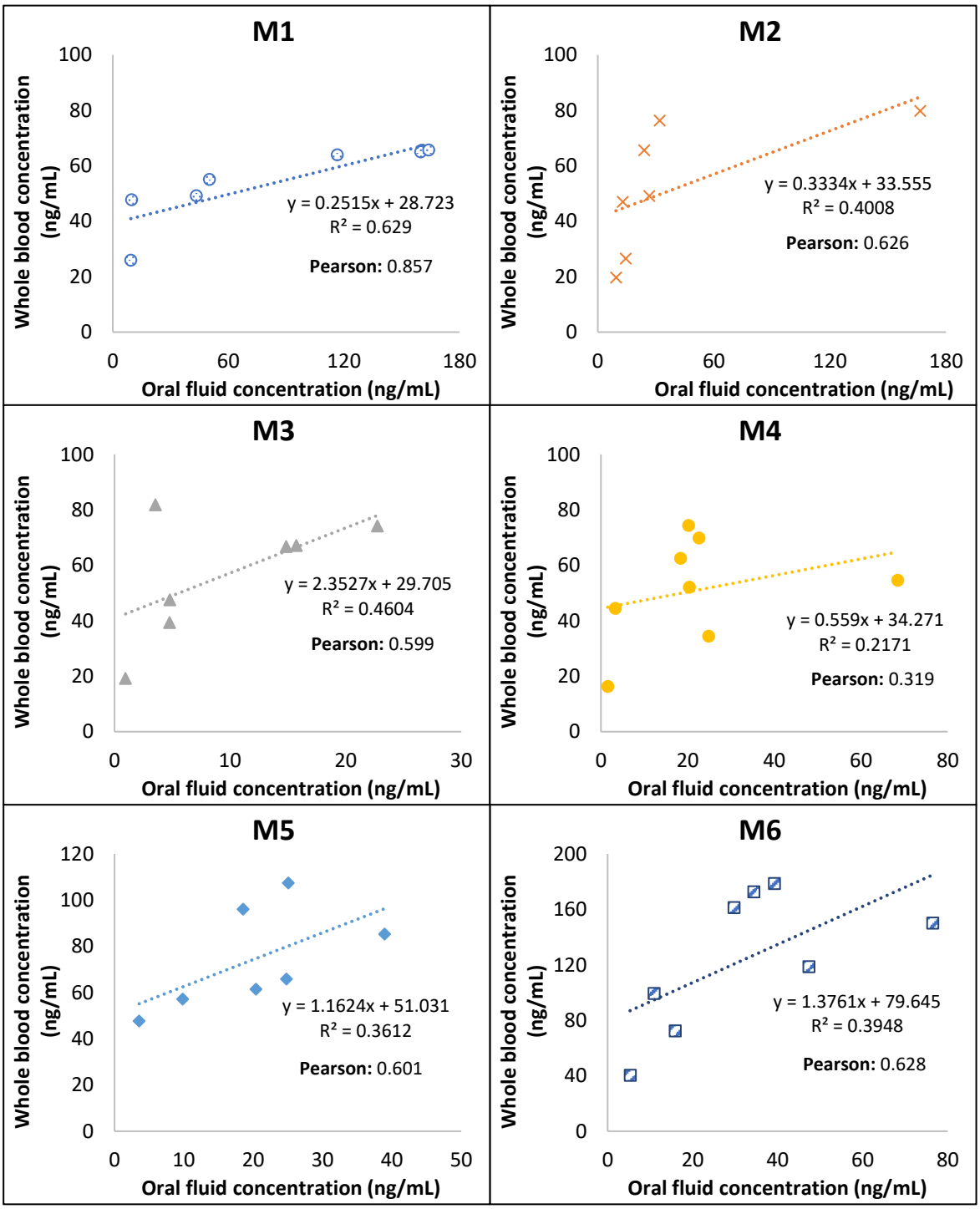


Figure 27. Correlation between oral fluid and plasma concentrations for **mephedrone** in M1-M6

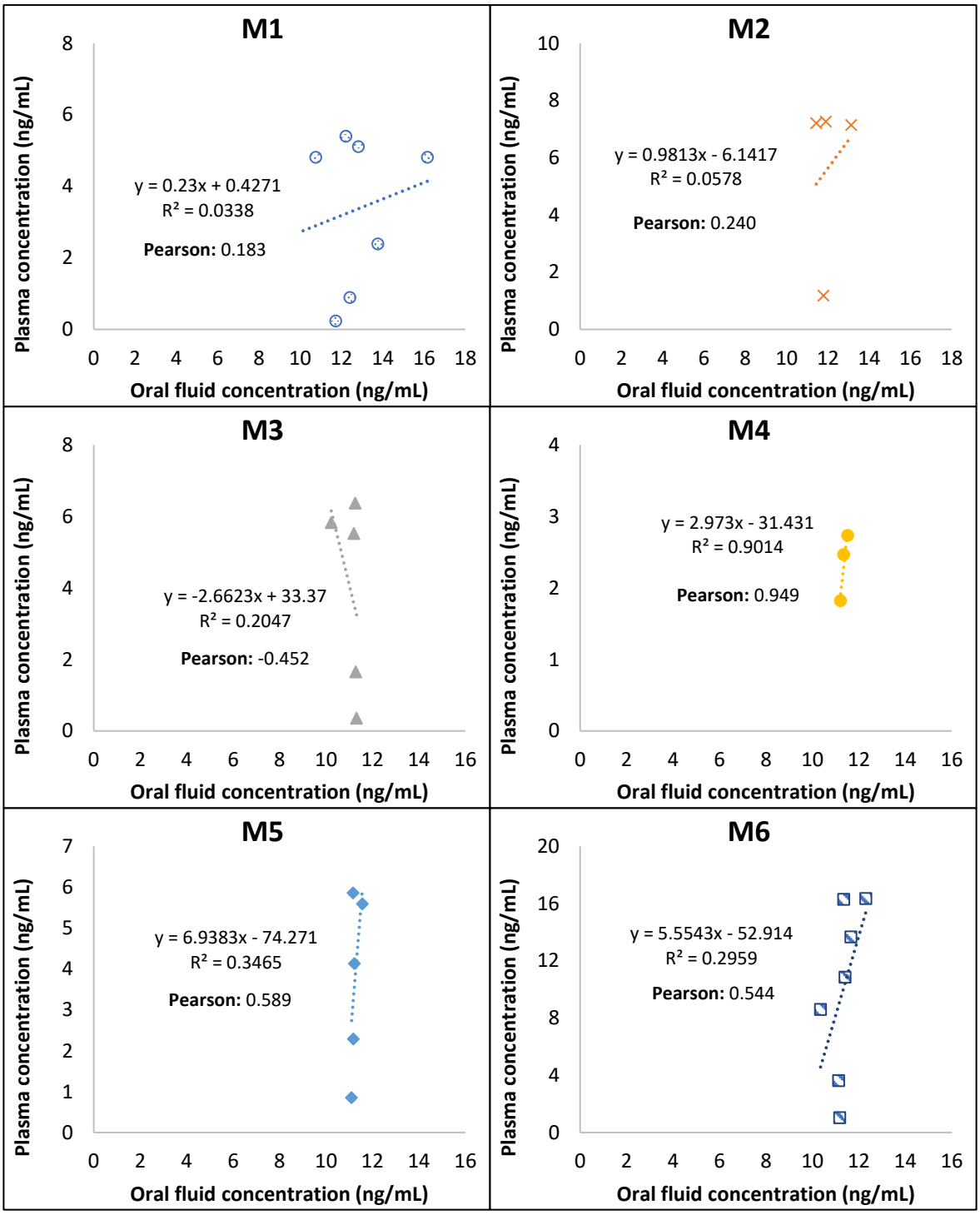


Figure 28. Correlation between oral fluid and plasma concentrations for **nor-mephedrone** in M1-M6